

NUTRITIONAL VALUE OF COLLAGEN IN MEAT PRODUCTS

Anita Laser Reuterswärd

Lund 1984



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LIST OF PAPERS

This thesis is based on the following papers, each referred to in the text by its Roman numeral (I-VI).

- I Laser Reuterswärd, A., Asp, N.-G., Björck, I. & Rudérus, H. (1982). Effect of collagen content and heat treatment on protein digestibility and biological value of meat products. *J. Food Techn.*, 17, 115-123.
- II Laser Reuterswärd, A. (1984). Solubilization of pigskin and bovine tendon after pepsin and pancreatin treatment. Effect of incubation conditions and age of animal. Submitted for publication.
- III Laser Reuterswärd, A., Asp, N.-G. & Björck, I. (1984). Protein digestibility of pigskin and bovine tendon in rats. Submitted for publication.
- IV Laser Reuterswärd, A. & Fabiansson, S. (1984). *In vivo* digestibility of insoluble collagen from bovine tendon as influenced by the inhibition of gastric acid secretion. Submitted for publication.
- V Laser Reuterswärd, A. & Björck, I. (1984). Urinary excretion of hydroxyproline in rats fed hydroxyproline-rich diets. Submitted for publication.
- VI Laser Reuterswärd, A., Andersson, H. & Asp, N.-G. (1984). Digestibility of collagenous fermented sausage in man. Submitted for publication.

ABSTRACT

This thesis deals with the nutritive value of collagen in meat products evaluated *in vitro* and *in vivo* (rats and humans). Highly collagenous materials, such as pigskin and tendon, were studied, both native and after heat treatment. The influence of animal age was also studied, since the intermolecular cross-linkages in collagen can change with animal age.

In vitro studies showed that the collagenous materials were highly solubilized and degraded after incubation with pepsin and pancreatin. Small but statistically significant differences in digestibility due to animal age were found *in vivo*, although both pigskins and tendons were all more than 92% digestible. The concentration of hydrochloric acid during pepsin incubation *in vitro* and gastric acid secretion *in vivo* were both found to be important for the digestion of insoluble tendon collagen but not for gelatin and meat proteins. Urinary hydroxyproline was excreted in free form by rats ingesting hydroxyproline-rich diets, but mainly as peptides when a collagenous sausage was consumed by man. However, only about 2% of the ingested hydroxyproline was excreted in the urine. In man, the collagenous fermented sausage was digested to the same extent as a reference sausage based on meat. In rats, a linear decrease of Net Protein Utilization was found when the amounts of collagen increased when mixed with meat proteins.

It is concluded that highly collagenous materials such as pigskin and tendon are digested *in vivo*, even without prior heat treatment and conversion to gelatin.

BASIC CONCEPTS AND ABBREVIATIONS

Digestion of proteins in the gastrointestinal tract is due to the action of several proteolytic enzymes: 1) pepsin in the stomach 2) trypsin, chymotrypsin, elastase, I and II, and carboxypeptidase A and B in the pancreatic juice, acting in the lumen of the upper small intestine 3) enzymes (peptidases) in the brush boarder membrane and in the cytoplasm of mucosal epithelial cells in the small intestine.

Intraluminal digestion results in free amino acids as well as peptides with the size of 2—6 amino acids. The final digestion of peptides to free amino acids occurs in the brush boarder membrane and the cytoplasm.

Pancreatin is an enzyme preparation containing pancreatic enzymes i.e. proteases, amylases and lipases.

Protein nutritional value is usually estimated by nitrogen (N) balance experiments **in vivo**. For collagen it can also be based on hydroxyproline (HPRO).

$$\text{True Digestibility, TD} = \frac{\text{N intake} - (\text{faecal N} - \text{metabolic N})}{\text{N intake}} \times 100$$

$$\text{Apparent Digestibility, AD} = \frac{(\text{N intake} - \text{faecal N})}{\text{N intake}} \times 100$$

Biological Value,

$$\text{BV} = \frac{\text{N intake} - (\text{faecal N} - \text{metabolic N}) - (\text{urinary N} - \text{endogenous N})}{\text{N intake} - (\text{faecal N} - \text{metabolic N})} \times 100$$

$$\text{Netto Protein Utilization, NPU} = \frac{\text{TD} \times \text{BV}}{100}$$

Metabolic N is the faecal N excreted by animals or man fed a protein free diet or a diet containing completely digestible protein.

Endogenous N is the urine N excreted by animals or man fed a protein free diet or a diet containing a protein that can be completely utilized for endogenous protein synthesis.

Protein Efficiency Ratio, PER, is another way to estimate protein quality. It is defined as g weight increase per g protein intake. PER is estimated during a four week experimental period.

The **collagen** molecule has three peptide chains forming a triple helix configuration. At both ends of the molecule non-helical peptides of less than 25 amino acids occur, being defined as **telopectides**. The main intermolecular cross-links are derived from the telopectides. A minor part of collagen present in tissues is **soluble** (free helix). The major part, the collagen fibres, consists of many helical molecules and is **insoluble**. It can be **solubilized** by enzymatic action or by heat treatment. **Denaturation** of the collagen helix results in three random chains, i.e. **gelatin**.

INTRODUCTION

Collagen constitutes about one third of the total body protein in mammals and is the major protein in skin, bone, tendon and cartilage (McClain, 1976). Intramuscular content of collagen in beef and pork varies between 2 and 20% (Lawrie, 1979) and the contents of protein of collagenous origin in meat products could be up to 35% (Fuchs & Kuivinen, 1980). Collagen is a fibrous, mainly insoluble, protein which is an integral component of connective tissue. It undergoes molecular changes as animal ages and has a characteristic amino acid pattern. Moreover, after heating, the collagen helix can be denatured and solubilized into gelatin which is of randomized structure and water soluble. Further heating does not render gelatin insoluble.

The enzymatic degradation of collagen has been reviewed from several different angles; gelatin manufacture (Balian & Bowes, 1977; Johns & Courts, 1977), leather industry (Gustavson, 1956), biochemistry and medicine (Weiss, 1976; Bailey & Etherington, 1980), tenderness of meat (Sørensen, 1976; Sørensen, 1981) and nutrition (Loewit, 1967; 1970; Brüggeman *et al.* 1964; Ashgar & Henrickson, 1982).

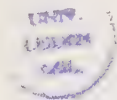
From the nutritional point of view the first criterion of protein quality is **digestibility**, being the amount of protein absorbed. The second criterion is the **biological value**, being the fraction of absorbed nitrogen retained in the body for maintenance and growth.

For nutritional labelling the protein content is based on the Kjeldahl nitrogen analysis. Thus both low digestibility and/or low biological value may make figures showing protein content misleading from the nutritional point of view.

Nutritional digestibility

The nutritive value of collagen in meat products has been a subject of discussion for quite a long time. Baumstark & Cohnheim (1910) were already discussing the question of connective tissue digestibility. They cited A. Schmidt who said 'Raw or smoked connective tissue cannot be digested by the intestine. If it has passed the pylorus unsolubilized it will be excreted in faeces'. Baumstark & Cohnheim themselves performed studies on dogs where they could not find any residues in the small intestine after meat pieces containing connective tissue were digested. The proposed explanation was that pepsin and hydrochloric acid digested the samples. Since then, many different studies with somewhat differing conclusions have been performed. Loewit (1967; 1970) reviewed the literature of collagen from the nutritional point of view and divided results concerning digestibility of raw collagen into three groups; **indigestible**, **difficult to digest** and **fully digestible**. A critical examination of the studies cited by Loewit, reveals the following:

Group 'Indigestible': a) Thomas & Seymour-Jones (1923) studied the trypsin action on collagen after treatment in different buffers at acidic pH and found hydrolysis values of about 80%. The authors did not discuss the question of digestibility and their system could not be considered as representative of the conditions that occur in the gastrointestinal tract. b) Möhler & Antonacopoulos (1957) investigated a method for determining collagen and pointed out that raw connective tissue is practically indigestible in man. However, they did not confirm this with any kind of experimental data.



Group 'Difficult to digest': a) Smorodinzew (1934) (Table 1), concluded that muscles with high amounts of collagen were 25-30% less digestible than muscles with low amounts of collagen. The *in vitro* system used, however, was largely unspecified and not related to *in vivo* conditions. b) Kotter (1957) discussed the collagen contents of different meat cuts containing connective tissue and pointed out its low content of essential amino acids and the resistance to proteolytic enzymes. He did not submit any kind of data on digestibility. c) Schönberg & Lochmann (1957) referred to Baumstark & Cohnheim (1910) but said that the latter's opinion that raw connective tissue is digested was optimistic and mostly not in accordance with others. Schönberg & Lochman themselves showed that connective tissue was resistant to trypsin treatment at pH 8 and this observation was proposed as usable as a method for evaluating amounts of connective tissue. Their *in vitro* study could not be considered representative for *in vivo* conditions. d) Grau (1960), investigated pepsin solubility of muscles from animals of varying ages but at the low temperature of 30°C, the meat having low amounts of connective tissue (Table 1). The conclusion by Loewit (1967) that Grau's data indicated 'difficult to digest' is not in fact substantiated by Grau. e) Franke (1964) showed that raw and cooked collagenous pigskin was 97% **digestible** *in vitro*, although not under well specified conditions (Table 1).

Group 'Fully digestible': a) Baumstark & Cohnheim (1910), found that pepsin solubilizes connective tissue proteins. However, solubility of collagen does not necessarily mean digestibility. b) Brüggeman *et al.* (1964) discussed several studies on the enzymatic digestion of connective tissue. The resistance of collagen to trypsin was explained by the authors as due to the absence of certain amino acids necessary for trypsin cleavage. The importance of pepsin and hydrochloric acid for the digestion of native collagen was pointed out and it was concluded that mammals can digest native collagen. It was also proposed that bacteria producing collagenase could digest native collagen in the intestine.

Some other *in vitro* studies performed where collagenous materials were incubated with pepsin, followed in some cases by pancreatin, are shown in Table 1. The concept used by different authors concerning solubility or digestibility is shown, as well as application of results to nutrition. Neuman & Tytell (1950) found tendons 95% solubilized and hide powder and kangaroo tail tendon were more than 80% solubilized. Lindner *et al.* (1965) found pigskin 95% digestible. Loewit (1967) on the other hand found raw pigskin and tendon 37 and 47% digestible but cooking increased these values. Etherington (1977) reported that the solubilization of bovine tendons in pepsin/acetic acid was dependent on animal age but anyhow above 65%. Harkness *et al.* (1978) found large differences in the solubility of rat tail and bovine tendons, 97 and 37%, respectively.

Opinions in more recently published literature are not well documented but it is generally considered that collagen is difficult to digest. Thus, Chefel (1977; 1979) indicated that unprocessed collagen is only partly digestible due to the presence of different types of cross-links. Kies (1981), discussing protein quality, pointed out that 'the earliest classification system of proteins was based on solubility of proteins, characteristics which have an impact on the digestibility. The fibrous proteins such as collagen, keratin and elastin tend to be insoluble, tough and resistant to digestion in contrast to globular proteins which tend to be fairly soluble and have high digestibility characteristics'. At the Swedish National Food Admin-

Table 1. Literature studies on *in vitro* solubility or digestibility of different collagenous materials

Sample	Collagen of protein or kind of preparation	Age of animal	Pepsin/hydrochloric acid			Pancreatin			Methodology			Concept	Nutr. applic.	Reference	
			Source	pH	Time h	Temp °C	Source	pH	Time	Temp	Weight %				Sol %
Meat 1st class	low		usp	1.6	3	37									
2nd class	high														
Stirloin	low	calf 4w and cow 14y	usp	1.2	0.5-6	30									
Shin	high														
Pigskin raw			usp	usp	usp	usp									
cooked															
Hide powder	extracted		usp	2.0	24	37									
Tendon:															
Achilles, cattle															
Achilles, pig															
Kangaroo tail															
Pigskin			usp	1.5	6	37	usp	8.4	6	37	96				
Pigskin, raw			Human gastric juice	2.7	3	37	Human gastric juice	8.0	14	37					
cooked															
Tendon, raw															
cooked															
Bovine Achilles	extracted	2y	swine	2.65	2	37									
Tendon		10y		acetic acid											
													</		

usp = unspecified

Dig = Digestibility; Sol = Solubility as used by the authors

Nutr. appl. = Nutritional application as used by the authors

¹⁾ Hydroxyproline solubility

²⁾ TCA = Trichloroacetic acid

istration, it was stated that 'some proteins (e.g. connective tissue and hair) cannot be digested in the stomach and have therefore no nutritive value' (von Hofsten *et al.* 1978). Rogowski (1980) indicated that in order to be made digestible, collagen must be heated wet, the heat treatment reducing the differences in the digestibility of different meats. Lawrie (1980), when discussing the nutritive value of offals mentions **digestible** collagen, thus indicating that some collagen is indigestible.

It is generally considered that collagen fibres are more resistant to degradation by proteolytic enzymes than most other protein fibres but are readily attacked by collagenase (FDA, 1978). Denatured collagen, however, can be attacked by a number of proteolytic enzymes (Paul, 1972; Ashgar & Henrickson, 1982). Ashgar & Henrickson (1982), when discussing nutrition, indicated that highly acidic conditions in the stomach ($\text{pH} < 2.0$) cause denaturation of collagen, making the triple helix susceptible to enzymatic digestion. However, no reference was given. Davidson *et al.* (1979) pointed out that muscle proteins are more rapidly and easily digested than collagen.

Thus opinions and facts concerning the digestibility of collagen are rather confused and contradictory. One obvious reason is that **enzymatic digestibility** i.e. *in vitro* digestibility (under conditions, not always representative for *in vivo*) is mixed up with **nutritional digestibility** i.e. *in vivo* digestibility. Another reason could be that the collagen investigated is not always specified with regard to origin, cross-links, animal age, particle size or heat treatment.

In vivo digestibility of collagen has in fact been investigated by very few authors. About 90% digestibility (apparent digestibility in rats) was found for limed cattlehide (Whitmore *et al.* 1975), partially defatted chopped beef with 50% collagen (Happich *et al.* 1975) and raw (probably scalded) pigs' ears (Vaughn *et al.* 1979). However, since the samples were either limed or partly heated the collagen could not be considered as being native. Harkness *et al.* (1978) found that tendons from rats tail, sheep and cartilage were 100, 87 and 65% absorbed, respectively, as evaluated by faecal hydroxyproline in rats. The study was performed for only three days using only two rats in each group. A report from the FDA (1981) referred to studies where collagen was treated with glutaraldehyd and thereafter 31% digested by rats. However, the report concluded that collagen consumed in the form of sausage casings appears to be digestible and that native collagen is digestible by rats and presumably by man.

Gelatin given to rats was shown to result in an accumulation of nitrogen in the intestinal contents, indicating slow or poor digestibility (Chen *et al.* 1962). However, Ashley & Fisher (1966) and Mauron (1973) found gelatin to be 90% digestible in rats.

NPU and PER

The protein quality of various materials of collagenous origin has been investigated in rat assays by analyzing the NPU but without regarding digestibility. Dvorak (1972) investigated NPU (carcass method) of different organs and meat cuts with collagen contents up to 45% of the protein. Decreased NPU values were found with increased hydroxyproline content. Bender & Zia (1976) reported a decreased NPU value of meat (carcass method) when the intramuscular collagen content was increased.

From the results of Franke (1964), who estimated weight gain and nitrogen consumed by rats fed pigskin and tendon, a calculation of PER-values shows a drastic decrease as the collagen content of the diets increased. *In vivo* digestibilities were not estimated but no differences in weight gain between raw and cooked materials were reported. This could imply an equal digestibility of raw and cooked samples. Raw and cooked pigs' ears (about 70% collagen of protein) had a PER-value of 0.6 (Vaughn *et al.* 1979). A PER-value of less than zero was obtained with cattle hide (80% collagen), increasing to a PER-value of 1.1 when only 50% collagen was present (Happich *et al.* 1975). Yu Bang Lee *et al.* (1978) reported PER-values of about 2 when 50% of the protein was collagen.

When using the methodology of NPU and PER, a decreased value with increased collagen content could depend on 1) a low digestibility of collagen 2) the unbalanced amino acid pattern of collagen or 3) a combination.

Biological value

Mitchell & Carman (1926), pointed out that the assumption that 'the protein in cheap cuts of meat is equal in nutritive value to the protein in expensive cuts of meat can only have reference to the digestibility. Any assumption that they are equal with respect to the biological value of their nitrogen is quite unfounded'.

The biological value of gelatin is expected to be low due to the complete absence of tryptophan (Bender *et al.* 1953) this also being true for collagen. However, the principle of complementation effects by combining proteins with different amino patterns is well known (Bressani, 1977). Thus, collagen itself can be considered a protein practically without nutritional value but could be mixed with other proteins, i.e. keratin, blood and casein, it could contribute with certain essential amino acids to the mixture (Chvapil, 1979). Kofranyi & Jekat (1969), for example, showed that in adults a mixture of muscle protein and gelatin, 1:1, had the same biological value as muscle proteins alone.

Collagen structure

Basic molecular structure

Collagen is a part of the intercellular matrix of connective tissue. Together with the other fibrous protein elastin, collagen is embedded in an amorphous ground substance. The properties of a particular tissue are determined by the relative proportion of each type of fibre and of other constituents such as glycosaminoglycans, water and calcium salts. The morphological arrangement of the collagen fibres vary in different tissues; in tendon they are parallel but in skin they are random (Bailey & Etherington, 1980).

Collagen fibres are built up from rod-like molecules with a diameter of about 1.5 nm and a length of 300 nm. The collagen molecule consists of three polypeptide α -chains bound in a triple helical configuration also called tropocollagen, the collagen monomer or the collagen fibril (microfibril). The α -chains are of two kinds, $\alpha 1$ and $\alpha 2$. At both ends of the molecule short, non-helical parts of the α -chains, called the N- and C-terminal telopeptides, are situated. The molecular weight of the collagen molecule is about 300 000 daltons (Bailey & Etherington, 1980; Etherington & Sims, 1981).

Different models exist concerning the three-dimensional structure of collagen molecules, as reviewed by Privalov (1982). In this context only the model presented by Bailey and co-workers is referred to (Bailey, 1982): The collagen fibrils are probably aligned two-dimensionally in a quarter-staggered fashion, to permit head to tail cross-linking between bonds derived from the N- and C-terminal telopeptide regions, i.e. intermolecular cross-links. In mature tissues, three-dimensionally pentafibrils are probably organized and arranged in register to permit interaction between the end-terminals. In this way a network of lateral and transverse cross-links to stabilize the fibre can be generated involving no other reaction than those of N- and C-terminal lysine-aldehydes. Presumably the transverse cross-link is a tri- or tetra-valent cross-link derived from the reducible aldimine and keto-imine cross-link (Fig. 1).

Since the early 1970s, five different genetic Types, I-V, of collagen have been identified, the Types occurring in different proportions in different tissues. Type I is heterogenous, as two $\alpha 1$ and one $\alpha 2$ polypeptide chains together constitute the tropocollagen $(\alpha 1(I))_2\alpha 2$, whereas Type II and Type III consist of three identical chains. Type IV has an unknown molecular composition and Type V two different chains. The nature of the different Types is characterized as: Type I fibrous; Type II and III fine fibres and Type IV an amorphous basement membrane, non-fibrillar. Type I collagen is the major component of tendon and bone (90%) and adult skin (80%). In muscles, the connective tissue is the framework surrounding the single muscle fibre (endomysium), the muscle fibre bundles (perimysium) and the entire muscle (epimysium). The major locations of different Types are: Type I in epimysium, Type III in perimysium and Type IV and V in endomysium (Bailey & Etherington, 1980; Sims & Bailey, 1981; Sørensen, 1981; Bailey, 1982; Bailey, 1983). The Types of collagen differ as to the level of amino acid sequences and peptide organization but the cross-linking mechanism and age effect are similar in different Types (Bailey, personal communication).

Amino acid composition

The amino acid sequence of the polypeptide chains is unique among proteins in that every third residue is glycine and that it possesses the regulary repeating tripeptide Glycine-X-Y. Glycine-Proline-Y and Glycine-X-Hydroxyproline occur in almost equal proportions (21% of the total amino acids). The entire sequence of the 1052 residues of e.g. the $\alpha 1$ (I) chain has been identified. However, none of the α -chains of collagen from a particular species have as yet been identified in their entirety, but work has progressed farthest in the case of calf skin collagen. The amino acid composition varies e.g. between different chains ($\alpha 1$ and $\alpha 2$) and between different Types of $\alpha 1$ (I and II) (Fietzek & Kühn, 1976; Bailey & Etherington, 1980; Sørensen, 1981).

The amino acid composition of the collagens (and their derived gelatins) is most characteristic and completely different from that of any other protein or group of proteins. The non-essential amino acids glycine, alanine, proline and hydroxyproline account for two out of three residues, so that only one third of the positions in the polypeptide chain is available for the remaining fourteen amino acids. The essential amino acids methionine, tyrosine and histidine are rare and cystine and tryptophan completely absent (Eastoe, 1967). However, small amounts of cystine have recently been identified in Type III and Type IV collagens (as referred to by

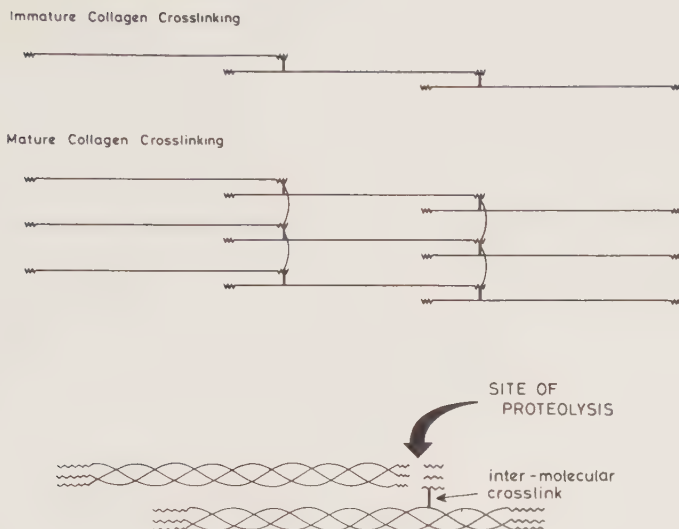


Fig. 1. Upper figure: Diagrammatic representation of the location of the cross-links. (a) In immature collagen the cross-links are restricted to the end-overlap region involving the telopeptide aldehyde; (b) in mature collagen the above intermediate cross-links form interfibrillar cross-links with molecules in register (Bailey, 1982).

Lower figure: Certain proteolytic enzymes (e.g. pepsin) will solubilize collagen by cleaving the molecule between the cross-link and the helical region (Sims & Bailey, 1981).

Sørensen, 1981). Cystine is then present in the C-terminal region, allowing disulphide bonds to occur and is probably involved in intermolecular bonds (Bailey & Etherington, 1980; Bailey, 1983). Calculations show that the total amount of the ten essential and semiessential amino acids is less than 20% for collagen (gelatin) compared to about 40% for non-collagenous muscle proteins (Table 3).

The content of 4-hydroxyproline of collagen is about 14% but varies between different Types; Type I - 13.1%, Type II - 15.0%, Type III - 17.4% and Type IV - 16.6% (expressed as free imino acid) (Etherington & Sims, 1981). Hydroxyproline is found in small amounts in only some other proteins such as acetylcholinesterase, a subcomponent of serum complement and elastin. Elastin, containing 1.5% hydroxyproline, is found in small quantities in skin, tendon and muscle (Johns, 1977; Bailey & Etherington, 1980; Etherington & Sims, 1981). Hydroxyproline determination is generally accepted as a method of estimating collagen (Etherington & Sims, 1981).

The hydroxylysine content, in contrast to 4-hydroxyproline, varies greatly from tissue to tissue and during animal ageing. Type I, e.g., contains 5-7 residues per 1000 in the helix. In Type I in skin collagen lysine is not hydroxylated to any significant extent in the telopeptide regions (Bailey & Etherington, 1980).

Telopeptides and cross-links

The N- and C-terminal ends of the α -chains (telopeptides) collagen consist of sequences of 10-25 residues, which are only partly determined, as e.g. for Type I collagen. Characteristically, glycine does not occur in every third position which is why this region is not in triple helical form (Piez, 1976; Bailey & Etherington, 1980). For the N-terminal telopeptide of $\alpha 1$ (I), all residues are known for calf and pig. All amino acids are determined for the $\alpha 2$ chain of calf but some are not known for pig. The C-terminal region of calf $\alpha 1$ (I) is mapped out (Piez, 1976), but not determined for pig.

The main intra- and intermolecular cross-links are derived from the telopeptides. The location of these cross-links in the N-terminal region originates from the lysine, occurring at residue 9 ($\alpha 1$) and 6 ($\alpha 2$). The latter residue number varies between species. The intramolecular bond, known as 'allysine', is known to occur in significant proportions of Type I collagen in the N-terminal region. The bond is a very stable cross-link between one of the $\alpha 1$ chains and the $\alpha 2$ chain, involving lysine residues. For the C-terminal region of Type I, the lysine situated at residue 1044 is the site for cross-linking. The two main intermolecular cross-links are the dehydrohydroxylysinoxynorleucine, 'aldimine', which is labile against weak acid and heat and the hydroxylysino-5-keto-norleucine, 'keto', which is stable against acid and heat. Of these two, the aldimine is the major cross-link in skin, about 98%, whilst tendon collagen has equal proportions of the two bonds (Weiss, 1976; Bailey & Etherington, 1980; Sims & Bailey, 1981). It is known that as an animal ages collagen contains less of the acid labile intermolecular aldimine cross-links; the cross-links being converted (stabilized) instead to an acid stable non-reducible form (not chemically identified) (Bailey *et al.* 1974; Sims and Bailey, 1981).

Soluble and insoluble collagen

Collagen fibres are known to be partly soluble, divided into neutral salt soluble and acid soluble fractions. The tropocollagen (helix) can thus exist intact in solution. During acid extraction, the salt soluble collagen will also be extracted (Johns, 1977). Neutral salt soluble collagen contains fewer cross-linked α -chains than any other form and acid soluble collagen contains a greater number of intramolecular cross-links as well as some intermolecular cross-links (Weiss, 1976). The amount of acid soluble collagen is known to depend on the species, age and technique used; Johns (1977) giving a value of about 10% for skin from different species, while Ashgar and Henrickson (1982) give about 3% for skin. Carmichael and Lawrie (1967) found a value of less than 10% for young and old tendon.

The major part of collagen in pigskin and tendon is thus insoluble.

Denaturation in solution and by heat treatment

From the nutritional point of view the denaturation of collagen after a specified heat treatment is of considerable interest as several authors indicate that collagen has to be converted to gelatin by heat before becoming digestible (Paul, 1972; Rogowski, 1980; Ashgar & Henrickson, 1982).

Collagen can be converted to gelatin partly in solution and partly by heat treatment. The denaturation temperature, T_m , where half of the helix collapses into random chains i.e. gelatinization, is clearly defined for **soluble** tropocollagen but dependent on the origin of the collagen. The thermostability of different

collagens has been shown to correlate with the presence of total imino acid content (proline plus hydroxyproline). Thus, e.g., skins and tendons from different species have T_m values of 36–41°C in saltfree solutions at pH 3.7 (Privalov 1982). The denaturation temperature is pH-dependent (Dick & Nordwig, 1966). Insoluble collagen fibres denature at their **shrinkage temperature** (T_s) which is 27°C $\pm$ 3° above the T_m of the corresponding tropocollagen (Bailey, 1968; Finch & Ledward, 1972). T_s is also pH-dependent, a sharp decrease being reported below pH 5 (Burge *et al.* 1958). The shrinkage temperature will rise if intermolecular cross-linking occurs (Bailey, personal communication). The shrinkage temperature of collagen fibres increases considerably (to more than 100°C) when the water content is decreased (e.g. to about 13%) (Finch & Ledward, 1972).

The denaturation process of collagen is relatively slow when compared with other proteins (Privalov, 1982). Bailey (1968) points out that denaturation in solution is a two-step process, the breakdown of the helical structure followed by dissociation of the polypeptide chains, which occur much slower. Sørensen (1981), when discussing tenderness, mentions four states between native collagen and gelatinized collagen.

Influence of animal age on collagen properties

The influence of animal age on collagen properties has been measured using different techniques. Isometric tension is one such technique where the collagen is not allowed to change in length and the force required to prevent shrinkage is measured at the thermal denaturation temperature. Large differences were found in isometric tension between tendons from an one month old animal (immature) and one of 18 months. This was also true for skin from a 3 week old rat compared to skin from an 18 month old one (Sims & Bailey, 1981). In both cases the maximal tension obtained occurred at about 65°C for the young material but at about 73°C for the older material. Intramuscular connective tissue (IMCT) of ox *M. sternomandibularis* (perimysial) developed higher isometric tension than the terminal tendon of the same muscle. This indicated that IMCT had ten to fourteen times the number of cross-links found in tendon collagen (Mohr & Bendall, 1969). Shear value of meat (toughness) increased when derived from a 7 year old cow compared to a 3 month old calf (Bailey, 1983). For calf skin, a direct relationship between amounts of reducible cross-links and solubility (water 80°C, 2 h) has been shown, reaching a maximum at about 1 year of age and with small decreases above about 4 years of age (Robins *et al.*, 1973). Hill (1966) reported decreased solubility of intramuscular collagen with increased age of animal (Ringer's solution, 63 min, 77°C); about 22% from 8–9 week old calves, 12% for 10 month old steers and only about 4% from 4 year old cows. This decrease in solubility is undoubtedly a reflection of the degree and type of cross-linking present in collagen (Sims & Bailey, 1981).

Enzymatic action on collagen

Pepsin has been used to confirm the concept of telopeptides and their contents of intermolecular bonds of collagen. It is now generally accepted that pepsin is not capable of disrupting the helical configuration of undenatured collagen but attacks only the telopeptide region (Weiss, 1976). Pepsin is therefore extensively used in combination with 0.5 M acetic acid for solubilizing collagen from a wide variety of

tissues, with the helical region intact (Sims and Bailey, 1981; Sørensen, 1981) (Fig. 1). The extent of solubilization with pepsin/acetic acid varies and most of the fibrous collagen could remain intact depending on conditions for incubation (Etherington, 1977). Moreover, acetic acid induces 50% greater swelling at pH 2 than that of hydrochloric acid (Gustavson, 1956). From the nutritional point of view it would be extremely important to get a high degree of solubilization and degradation for obtaining a high digestibility of collagen.

Chymotrypsin, trypsin, elastase and lysosomal cathepsins also cleave the undenatured collagen molecule only in the non-helical telopeptide region, leaving the helix intact (Weiss, 1976; Bailey & Etherington, 1980) but the cleavage occurs at different sites by different enzymes (Drake *et al.* 1966).

Collagenase, by definition, exhibits its hydrolytic action at specific peptide bonds in the triple helix, which is totally resistant to all other known proteinases and collagenase has a maximum activity at or near pH 7.5. Bacterial collagenases degrade native collagen to small dialysable peptides, but vertebrate collagenase cleave all the three peptides in the helix at a single locus (Bailey & Etherington, 1980).

AIMS OF THE INVESTIGATION

This investigation deals with the following questions concerning collagen and nutrition.

- * Are highly collagenous materials such as pigskin and tendon digestible *in vivo*?
- * Which factors in the gastrointestinal tract are especially important for the digestion of collagen?
- * Can pepsin and/or pancreatin solubilize and degrade collagen *in vitro*?
- * Is hydrochloric acid concentration and pH of gastric acid secretion critical for the digestion of collagen *in vitro* and *in vivo*?
- * Is the digestibility of collagenous materials influenced by a) prior heat treatment such as scalding and cooking b) animal age?
- * Is *in vitro* solubilization of collagen an indication of digestibility *in vivo*?
- * Are rat assays relevant in predicting protein nutritional value for man?
- * What is the nutritive value of collagen in combination with meat proteins?

RESULTS AND DISCUSSION

Digestibility

Prior heat treatment

Paper I shows the effect of heat treatment on true digestibility in rats using a mixture of pigskin and meat (50/50) at collagen contents of about 40%. Samples that were raw, heated wet to 74°C for 30 min and to 95°C for 30 min all had a digestibility of 99.2%, pure meat being 100% digestible. Thus, the admixture of collagenous materials and heat treatment had no effect on the digestibility. The raw sample, however, was actually scalded according to slaughterhouse procedures. Therefore, pigskins from two unscalded pigs, one 4 months old and one 5 years old were tested (without the admixture of meat) but these samples were also highly digestible, 96.1% and 92.5%, respectively. Scalding had no significant effect on digestibility when two young pigskin samples were compared, both being more than 96% digestible (Paper III). In paper V another scalded pigskin was found to be 98% digestible. Altogether six pigskin samples, heat treated to 74°C for 30 minutes, were shown to be more than 95% digestible (I, III).

The scalding of pigs may denature **soluble** collagen in immature animals (Johns, 1977). However, more than 90% of skin collagen is insoluble (Johns, 1977) and denatures at about 65°C (Bailey, 1968). The fraction of denatured collagen in the pigskins (from scalded pigs) used would therefore have been negligible. However, after heat treatment at 74°C large parts of the pigskin collagen would have denatured. From the results obtained, prior heat treatment was not necessary; native as well as scalded pigskins being highly digested in the rats.

Age-effect

In paper III the digestibility of pigskin and tendon from animals of varying ages was studied in rats. True digestibilities of proteins in young, (4 month) unscalded pigskin were 96.1% and old (5 year), unscalded pigskin 92.5%. Proteins in calf tendon (7 month) were 97.3% digestible and cow tendon (6.6 years) 92.4%. Small but statistically significant differences due to animal age were thus found. Wet heat treatment of the old pigskin and cow tendon samples, at 74°C for 30 min, significantly increased the digestibilities to 96.3 and 97.3%, respectively.

It is known that as animals age, collagen fibres become more stable as assessed by tensile strength, isometric tension, swelling and solubility in hot water and acid. This is due to the age stabilization of intermolecular cross-links (Weiss, 1976; Sims & Bailey, 1981; Bailey, 1982). Increased resistance to digesting collagen from mature animals by pepsin has been reported (Weiss, 1976). Decreased solubilization by pepsin/acetic acid for insoluble collagen from old animals was found (Etherington, 1977). The present *in vivo* study indicated that despite an increase in age, high digestibilities were obtained under conditions occurring in the gastrointestinal tract of rats. In contrast, when **artificial** cross-links were introduced, the *in vivo* digestibility of rat tail tendon was reported to decrease from 102% to 39% (Harkness *et al.* 1978).

Collagen matrix

The true digestibility of extracted insoluble collagen (bovine tendon) was compared to calf and cow tendon samples. A value of 95.2% was found i.e. a value between those of calf and cow tendon proteins (96% collagen) but not significantly different from either of them. Etherington (1977) pointed out that proteoglycans and glucosaminoglycans present in the collagen matrix, (mainly extracted in our sample) may have a stabilizing role complementary to the intermolecular cross-links of collagen and that these carbohydrates may restrict access of an enzyme to the non-helical telopeptide region. However, the present findings indicate that the presence of the tissue matrix of calf and cow tendon did not limit digestion *in vivo*. This was also true for the pigskin samples, which all had a high digestibility.

Enzymatic solubilization and degradation

Paper II deals with enzymatic solubilization and degradation of collagenous pigskin and tendon from animals of different ages. The *in vitro* assay was usually performed using pepsin for 2h and/or pancreatin for 2h, at 37°C.

Acid solubility. Young (4 month) and old (5 year) pigskin samples were 20% and 5% soluble, respectively, in hydrochloric acid, pH 1.5, as assessed by hydroxyproline analyses. The value for young pigskin is somewhat higher, but that of old pigskin is equal, to the values of solubility in weak acid reported for skin in general by Johns (1977) and Ashgar & Henrickson (1982). The pigskin material also included about 25% non-collagenous proteins which were found as not being totally soluble in acid. The age-effect obtained was probably due to the fact that the acid labile intermolecular cross-link ('aldimine') is converted into a stable form due to animal age (Bailey *et al.* 1974; Sims & Bailey, 1981).

Most of the collagen present in pigskin was thus in insoluble form and had to be solubilized by enzymes. This would also be true for tendon samples, since the collagen fibres in tendons are mainly insoluble (90%) (Carmichael & Lawrie, 1967).

Swelling. Pancreatin alone only solubilized the nitrogen of the young and old pigskin samples to a small extent, 12-15%. However, after pre-swelling of the samples in hydrochloric acid (pH 1.5) the nitrogen solubilities increased to 60% for young and 28% for old samples. Swelling has been indicated as being necessary for expanding the interfibrillar space of collagen making it susceptible to enzyme action (Bailey & Etherington, 1980). A tighter structure present in collagen from old animals (Bailey, 1982) could have resulted in a less efficient swelling of the old pigskin sample.

Solubilization by pepsin and pancreatin. Pepsin, at pH 1.5, solubilized young and old pigskin samples to a higher extent than pancreatin after pre-swelling. The young sample was 84% and the old 73% solubilized. Further treatment with pancreatin solubilized the materials to 92% and 76%, respectively. Tendon samples were also shown to be highly solubilized in pepsin at pH 1.5. Hammer-milled samples were about 86% solubilized (nitrogen). Pancreatin increased the solubilities to about 91%. No significant age-effect was found for tendons.

Degree of degradation. Trichloroacetic acid (TCA) was used as a precipitation agent in order to separate protein from peptides after enzymatic degradation. A high concentration (25% w/v) was found to be necessary after pepsin digestion.

The TCA-solubilities after pepsin incubation were less than 10% for all pigskin and tendon samples, as well as gelatin, independent of age and heat treatment. This can be explained by the fact that pepsin is cleaving only at sites of tyrosine-aspartic acid and glycine-valine in gelatin (Weiss, 1976). These amino acids occurred in very small amounts in the pigskin and tendon samples used (III) and in gelatin (Table 3). The TCA-solubilities after pepsin/pancreatin treatment showed no significant difference from values without TCA-precipitation, thus all the collagen (protein) that was solubilized was also degraded. Pancreatin is known to cleave at many more sites in gelatin (Weiss, 1976) which could explain the high TCA-solubility obtained.

In vitro - in vivo correlation

Samples that were analyzed both *in vitro* (TCA-solubility) and *in vivo* (true digestibility, TD) are shown in Table 2. The young scalded sample had a bigger particle size when used *in vitro* than *in vivo*, which could probably partly explain the lower solubility obtained. The age-effect, which resulted in lower solubility as well as lower TD when young and old pigskins are compared, was much more pronounced *in vitro*. The results show that *in vivo* digestion was more effective than the *in vitro* solubilization, as designed in paper II.

Table 2. *In vitro* and *in vivo* results of the solubility and the digestibility of pigskin and tendon samples. Wet heat treatment was performed at 74°C for 30 minutes. HM = hammer-milled.

	In vitro ¹⁾ TCA-solubility (pepsin/pancreatin) %	In vivo ²⁾ True digestibility %
<u>Pigskin</u>		
Young (4m) unscalded	94.2	96.1
Young (4m) heated	97.0	97.4 (HM)
Young (6m) scalded	82.6	97.5 (HM)
Young (6m) scalded/heated	96.5	97.7 (HM)
Old (5y) unscalded	75.2	92.5
Old (5y) heated	81.4	96.3 (HM)
<u>Bovine Achilles Tendon</u>		
Calf (7m) raw, HM	92.4	97.3
Cow (6.6y) raw, HM	91.9	92.4
Cow (6.6y) heated, HM	92.8	97.8
Insoluble collagen	92.5	95.2
Gelatin	100.0	—
Gelatin/whole egg	—	99.4
0.78/0.72		
Meat	100.2	99.8

¹⁾ *In vitro* results from paper II; TCA = trichloroacetic acid

²⁾ *In vivo* results from paper III, except gelatin/whole egg (IV) and meat (I)

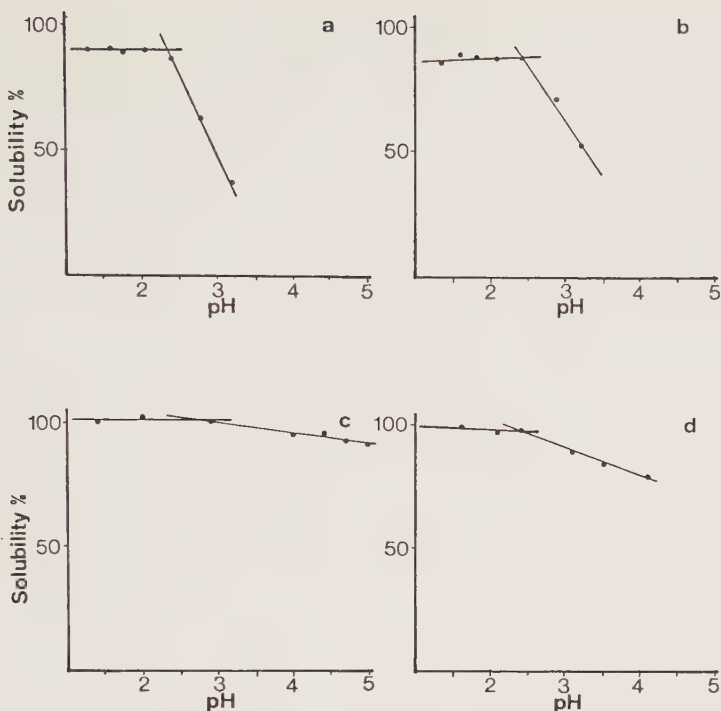


Fig. 2. Nitrogen solubility, as a % of total nitrogen in sample. Sample (a) insoluble collagen, (b) insoluble collagen, (c) gelatin and (d) meat. Incubation for 2 h at 37°C with pepsin in hydrochloric acid at different pH levels. In samples (b), (c) and (d) this was followed by incubation in pancreatin for 2 h at 37°C and precipitated by trichloroacetic acid (25 % w/v final concentration). $n=3$, standard deviations less than 6.0.

pH during pepsin digestion

In the *in vitro* study (II) the influence of varying pH during pepsin incubation was examined. It was shown that already at a pH of 2.0-2.5 the nitrogen solubility of old (5 year) unscaled pigskin (II) and for young (4 month) unscaled pigskin (not shown in II) decreased drastically. This was true also for insoluble tendon (Fig. 2). Further treatment with pancreatin had only a small solubilizing effect. However, for gelatin and meat proteins this pH-effect was not that pronounced (Fig. 2).

In order to test the influence of gastric acid secretion *in vivo* it was inhibited in rats using a drug, omeprazole (IV). Rats were given insoluble tendon collagen, gelatin or meat proteins. The true digestibility of collagen was found to be 71%, which was significantly lower than the value of 95% obtained without inhibiting gastric acid secretion. Gelatin and meat proteins were not affected by the inhibition, both being more than 98% digested.

Both the *in vitro* and *in vivo* results thus indicated that hydrochloric acid (pH) has a much greater influence on the digestion of insoluble collagen than of gelatin and meat proteins.

Digestibility of collagenous sausage in man

No previous study is available where the digestibility of collagen or gelatin has been directly evaluated in man. In paper VI a fermented collagenous sausage was given daily to man in four ordinary meals. The protein intake was about 64 g daily, of which 85% was derived from the sausage. Almost half of the total protein was from pigskin collagen derived from scalded pigs. A reference sausage based on meat was also given. Three ileostomists participated, serving as their own control. True digestibilities (nitrogen) were found to be 71-79% for the collagenous diets and 69-85% for the reference diets. Hydroxyproline digestibilities (apparent digestibility and true digestibility) for the collagenous diet were 70-82%. This indicated a similar digestibility of collagen and meat proteins. Amino acid patterns of ileal excreta distinctly differed between the two sausages, confirming that not all the proteins were absorbed. However, the ileostomists were considered as having some malabsorption due to ileal resection which could explain why both the reference and collagenous sausages did not show even higher digestibility values. In rats meat proteins are digested to more than 96% (Mauron, 1973; I) and fermented sausage to 92% (Eskeland & Nordal, 1980).

It was concluded that in man, fermented collagenous sausage is digested to the same extent as a reference sausage based on meat proteins. The main part of the collagen present was considered as insoluble and not affected by the processing (scalding and fermentation). The study thus confirmed that collagen in pigskin is highly digestible in man even without prior conversion to gelatin by heat treatment.

Urinary excretion of hydroxyproline in rats and man

Two aspects of the collagen molecule could be especially critical for degradation and digestibility. Firstly, insoluble collagen has to be solubilized before denaturation and enzymatic hydrolysis. Secondly, the unusual amino acid pattern of denatured collagen (gelatin) could make it a poor substrate for exo- and endopeptidases, resulting in large fragments not easily susceptible to further enzymatic attack (Weiss, 1976). Several studies indicate that hydroxyproline peptides can be absorbed and excreted intact in the urine in rats and man (Hueckel & Rogers, 1970; Prockop & Sjoerdma, 1961; Prockop & Kivirikko, 1968). This would imply a decreased utilization of amino acids as a nitrogen and energy source.

In paper V the urinary excretion of hydroxyproline was studied in rats given casein, meat and hydroxyproline-rich diets as including gelatin, insoluble collagen and pigskin as the main protein source in nitrogen balance experiments. Elevated excretions were found after ingestion of the hydroxyproline-rich diets. The difference between total and free amounts was similar with all diets, indicating that the excretion of hydroxyproline containing peptides did not change. This finding is not in accordance with the studies referred to above, but might be explained by a difference in the hydroxyproline-rich protein dosage given to the animals. As discussed in paper V it would appear that there are dipeptidases which can hydrolyze peptides containing e.g. proline and hydroxyproline in the rat intestine, but these enzymes have relatively low activity.

Two persons studied in paper VI collected their urine after ingesting the fermented collagenous and reference sausages. Elevated amounts of hydroxyproline were found in the urine after consumption of the collagenous sausage, and

74% and 91% of the increase was as peptide bound hydroxyproline. This is in agreement with earlier reports for humans ingesting gelatin (Prockop & Sjoerdsma, 1961; Prockop *et al.* 1962; Bronstein *et al.* 1966). Those peptides so far identified as being excreted by humans consist of proline, hydroxyproline and glycine (Meilman *et al.* 1963). Hitherto no essential amino acids seem to have been identified in the excreted peptides after ingestion of gelatin.

For the rats ingesting hydroxyproline rich diets (V) no more than 2.5% of the ingested hydroxyproline was excreted intact as hydroxyproline (free). For the humans (VI) about 2% was excreted (peptide-bound). Both these values were lower than reported by Prockop *et al.* (1962) who found that 7% of the hydroxyproline in gelatin was excreted as hydroxyproline peptides in the urine. The urinary losses obtained in the present study do not therefore substantially diminish the utilization of amino acids as a nitrogen and energy source.

Factors important for the digestion of collagen in the gastrointestinal tract

From the results of the present investigations together with those in the literature, it can be proposed that five factors could be especially important for the digestion of insoluble collagen (i.e. collagen that has not denatured before consumption) and acid soluble collagen.

Firstly, hydrochloric acid can break the **acid labile intermolecular cross-links** of collagen. However, the degree of cleavage is probably relatively small since less than 10% of the collagen is acid soluble in e.g. skin and tendon (Johns, 1977; Carmichael & Lawrie, 1967).

The second factor is that of **swelling**, which has been shown to reach a maximum at pH 2 for e.g. hide collagen (Gustavson, 1956). Swelling has been indicated as being necessary for the action of enzymes in the interfibrillar space of collagen (Bailey & Etherington, 1980). Since the action of pancreatin and elastase is known only to occur in the non-helical telopeptide region of insoluble collagen (Weiss, 1976; Bailey & Etherington, 1980), this action would be weak at the neutral pH found in the small intestine of rats and humans. Collagenases are the only enzymes cleaving collagen in the intact helix and acting at neutral pH (Bailey & Etherington, 1980). However, Fullmer, *et al.* (1966) stated that they could not find any collagenolytic activity in the alimentary tract of rats. However, collagenolytic activity was reported in the gastrointestinal tract of 7 humans, and proposed to be related to ulceration. Fourteen other individuals did not show this activity (Riley & Peacock, 1967). Brüggeman *et al.* (1964) proposed that bacterias producing collagenase are important for digestion in man while Harkness *et al.* (1978) pointed out that this was not important for rats.

A third factor of hydrochloric acid is to provide an **optimum pH for pepsin activity** for degradation of collagen fibres. Pepsin action on undenatured collagen is known to occur only in the non-helical region (Weiss, 1976). Pepsinogen is converted to pepsin in solutions which are more acidic than pH 5, and the optimal pH of pepsin is around pH 2 for most proteins (Taylor, 1968). However, several mammalian gastric proteinases have been found; pepsins (e.g. pepsin 1 and 3), gastricsins (e.g. parapepsin II) and minor components (e.g. parapepsin I) (Foltman, 1981). Degradation of fibrous bovine tendon collagen by human pepsin 1 and 3 at 37°C has been reported (Etherington *et al.* 1980). Pepsin 3 showed a broad plateau of activity from pH 1.2 to 3.8 with an indistinct maximum at pH 3 to 3.5,

pepsin 1 showing a clear maximum near pH 3. Both pepsins were confirmed as cleaving the collagen molecule only in the telopeptide region. Human pepsins 1 and 3 are secreted in fundus and are predominant among the gastric proteases (Foltman, 1981). Pepsin 1 normally has lower activity than pepsin 3 (Etherington *et al.* 1980). *In vitro* experiments with human and porcine gastric juice have shown proteolytic activity with maxima at pH 2 and pH 3.5. The second maximum is due to the occurrence of parapepsin II, which probably plays an important role in the normal intragastric digestion of proteins (Taylor, 1959; Taylor, 1968; Ryle & Porter, 1959). This enzyme is secreted in fundus, antrum and proximal duodenum (Foltman, 1981). No data are available on the action of this enzyme on insoluble collagen. However, human gastric juice showed two pH maxima for the digestion of two blood proteins. For egg protein on the other hand only one maximum at pH 1.5 was recorded (Taylor, 1959). Parapepsin I, has shown high activity for gelatin (Ryle & Porter, 1959) and is probably equivalent to gelatinase (Etherington & Taylor, 1967). Gelatinase is characterized by 'an almost 450 times greater rate of hydrolysis of gelatin when compared to pepsin' (Northrop (1931) as cited by Gitler, 1964). This enzyme does not have a high activity against collagen (Steven, as cited by Ryle, 1964). It is therefore possible that gastric proteinases occur which are also active at pH higher than 2 and that such enzymes could be a critical factor in the solubilization of insoluble collagen.

It is noteworthy that pepsin is known to cleave at the specific sites of glycine-tyrosine-aspartic acid-glutamic acid or alanine-glycine-valine-alanine in gelatin (Weiss, 1976). In the N-terminal α 1-chains of calf and pig, as shown by Piez (1976), it should be impossible for pepsin to cleave at the sites necessary for separating of the intermolecular cross-linkages. In the C-terminal region of calf skin this seems more probable since a glycine-tyrosine-aspartic acid-leucine sequence exists closer to the helix than to the site of cross-linked lysine. Cleavage of the C-terminal region of pigskin and cow tendon collagen could be possible, providing that this sequence occurs in these collagenous tissues. The high degree of pepsin solubilization and of *in vivo* digestibility obtained in the present studies (I, II, III, IV, VI) could be the result of a more nonspecific cleavage of the peptide bonds in the non-helical regions than those mentioned by Weiss (1976).

Denaturation of collagen fibres can only occur after solubilization of the monomers (Etherington, 1977). The fourth factor of hydrochloric acid on the degradation of insoluble collagen could therefore be that of denaturation (gelatinization) of pepsin solubilized monomers (tropocollagen). The denaturation temperature of tropocollagen is around 39°C for mammalian collagen according to Bailey (1983). The denaturation temperature for different tropocollagens varies (Bailey, 1968; Privalov, 1982). The exact denaturation temperatures for bovine tendon or pigskin, which were used in the present study, are not available in the literature. However, the denaturation temperature is pH dependent and decreases at a lower pH as shown by Dick & Nordwig (1966). Their results on soluble calf skin showed a sigmoid curve, decreasing from 39°C at pH 5 to 32°C at pH 1 to 2. Body temperature is 37 to 38°C for rats (Falkmer & Waller, 1972) and man. It is therefore probable that hydrochloric acid lowers the denaturation temperature of the pepsin solubilized monomers below the body temperature.

The fifth factor is the final degradation of denatured chains of collagen by the action of **pancreatic** and **intestinal enzymes**, as for other proteins. In the *in vitro*

study (II) of insoluble collagen and pigskins it was shown that all collagen solubilized by pepsin at differing pH was further degraded into trichloroacetic acid soluble peptides by pancreatin. When studying hydroxyproline excretion (V) no evidence was found for a larger excretion of peptides after the inhibition by gastric acid of insoluble collagen (digestibility 71%) than for gelatin (digestibility 98%). In paper VI it was shown that in spite of distal small bowel resection in the ileostomy patients, the collagenous sausage was highly digested. This indicates that swelling and the solubilization by gastric proteinases in stomach, are probably the most critical for the digestion of insoluble collagen.

Amino acid composition

Amino acid composition of the materials used in the present study is shown in Table 3. The amino acid composition of the different pigskins was similar (III). No data on the complete amino acid composition of intact pigskin are available in the literature. However, when compared to results obtained by Eastoe (1967) on pure gelatin (and our results shown in Table 3) the contents of glycine, proline and hydroxyproline were lower in pigskin. In contrast considerably higher amounts of cystine, methionine, and tyrosine were found in the intact pigskin. The tryptophan

Table 3. Amino acid compositions of pigskin, insoluble tendon collagen, gelatin, meat and egg from different papers. Amino acids expressed in g/18gN, corresponding to 100 g protein.

	Pigskin ¹⁾ g/18gN	Insoluble tendon collagen ²⁾ g/18gN	Gelatin ³⁾ g/18gN	Meat ⁴⁾ g/16gN	Egg ⁵⁾ g/16gN
Aspartic acid	7.3	6.3	6.7	9.0	10.0
Threonine	2.4	2.0	2.1	4.6	5.0
Serine	4.4	3.6	4.1	4.2	8.1
Glutamic acid	12.0	10.6	11.1	15.2	12.7
Proline	14.4	13.9	13.9	3.8	3.6
Glycine	24.0	25.4	25.8	4.3	3.3
Alanine	10.1	10.4	9.7	5.6	5.6
Cystine	0.38	trace	0.14	1.2	2.6
Valine	3.2	2.6	2.6	4.8	6.5
Methionine	1.6	0.97	1.1	2.9	3.4
Isoleucine	1.8	1.6	1.5	4.6	5.2
Leucine	4.1	3.3	3.3	8.0	8.6
Tyrosine	1.3	0.69	0.69	3.4	4.2
Pheynlalanine	2.6	2.1	1.9	3.8	5.2
Histidine	1.1	0.76	0.96	3.6	2.5
Lysine	4.4	3.9	4.1	8.7	7.2
Hydroxylysine	0.89	2.6	1.5	zero	zero
Arginine	9.3	9.0	9.2	6.5	6.8
Ammonia	0.95	0.80	1.0	1.3	1.4
Hydroxyproline	10.4	13.6	12.6	0.34	zero
Tryptophan	0.15	zero	zero	1.1	1.5

¹⁾ From a 6 months scalded pig (II, III) (see also paper III for other pigskins)

²⁾ Extracted from bovine Achilles tendon (II, III, IV, V) (see paper III)

³⁾ From swine skin (II, IV, V)

⁴⁾ Beef, *M. psoas major* (IV, V)

⁵⁾ Whole hen's egg (IV, V)

content was about 0.15 g/100 g protein in pigskins but this amino acid is absent in gelatin. The amino acid composition of different tendon samples was similar (III) and in good agreement with the pattern reported for cattle Achilles tendon by Eastoe (1967). In the tendons, the content of lysine was considerably lower, and that of hydroxylysine higher, than in pigskins. However, the sum (g/100 g protein) of lysine and hydroxylysine was fairly constant, in pigskins 4.8 to 5.3 and in tendons 4.8 to 5.5, very much in line with the findings of Eastoe (1967).

Biological value and NPU

The biological value and NPU of different mixtures of pigskin and beef was studied in paper I. Since the digestibility of pigskin protein was high, NPU-values mainly reflect the biological value. An inverse relationship was found between NPU (y) and the collagen content of the protein mixture (x), according to linear regression: $y = 82.8 - 0.6x$ (correlation coefficient of 0.99) (Fig. 3). Linear extrapolation to 100% collagen gave a NPU of 23. This is in accordance with the concept that BV approaches 20 when chemical score for tryptophan is zero (Bender, 1973). According to Münchow and Bergner (1967) as cited by Bressani (1977), gelatin has a biological value of 8.2. The present results are also in accordance with those of Bender & Zia (1976) who showed a NPU value of 69 for shin with 23.6% collagen and a NPU value of 82 for fillet with 2.5% collagen. NPU values of 69 and 81%, respectively, can be deduced from the equation above for these contents of collagen. Corresponding NPU values of 65 and 83%, respectively, could be deduced from the study by Dvorak (1972) for twelve meat cuts and organs. The agreement between the NPU-values from these three studies could indicate that food collagen is generally well digested.

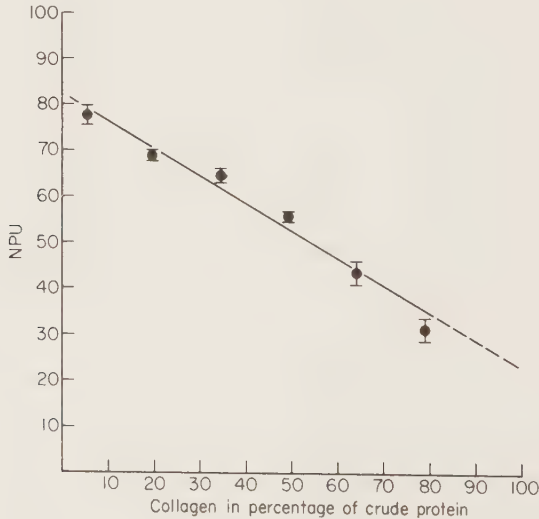


Fig. 3. Relationship between NPU-value and collagen content in mixtures of *M. biceps femoris* from beef and pigskin (mean values and s.e. mean). (I).

In paper V, biological value of insoluble collagen/egg protein and gelatin/egg protein (52%/48%) was studied. The insoluble collagen mixture had a biological value of 80.5% compared to that of gelatin mixture, 72.5%. A lower true digestibility of the insoluble collagen sample (71%) compared to the gelatin sample (99%) was obtained due to inhibition of gastric acid secretion, while the egg protein was fully digestible (IV). The difference in biological value was thus due to a higher proportion of absorbed amino acids from egg in the mixture of insoluble collagen/egg than in the mixture of gelatin/egg. The NPU-values of these mixtures, however, were the same, 69.1% and 70.7%, respectively. This shows that with the design of this experiment the higher digestibility obtained for gelatin was of no use for the rats since they could not use the amino acid surplus absorbed from gelatin.

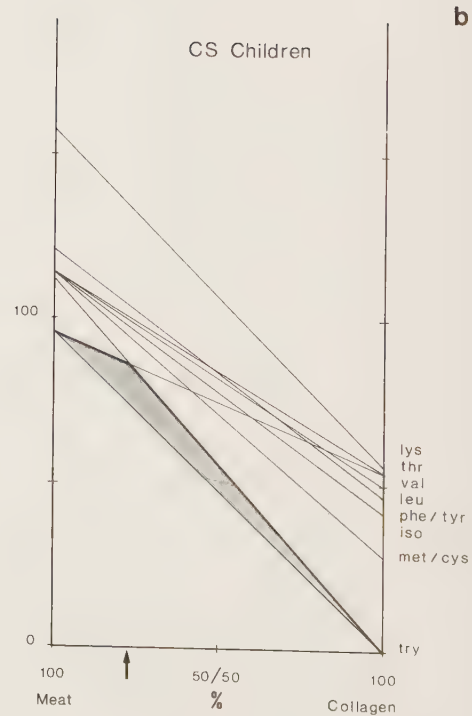
Complementation and supplementation

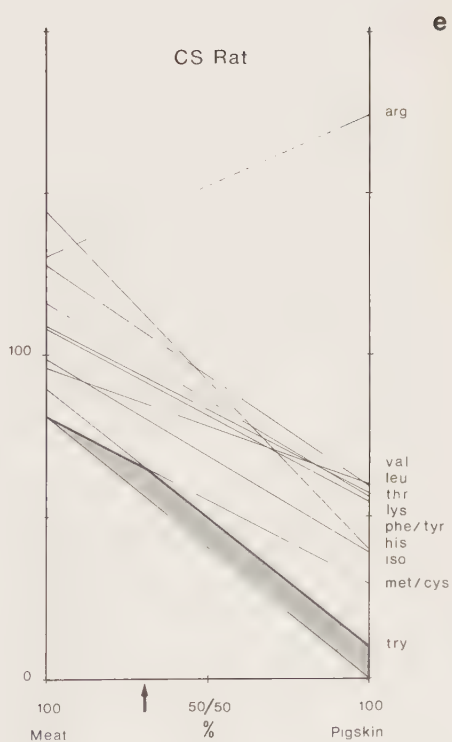
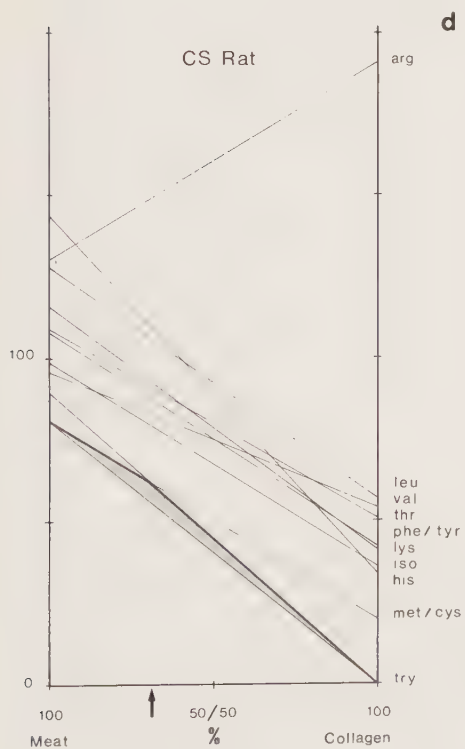
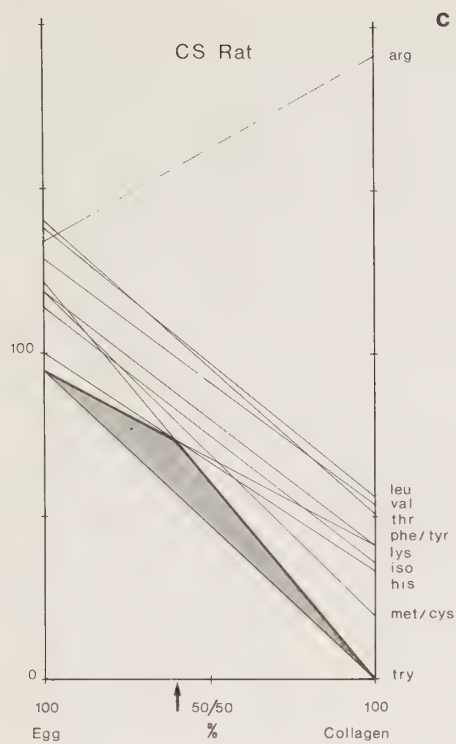
Collagen (gelatin) is a unique protein in that there is a complete absence of one essential amino acid, tryptophan. However, the complementary effect of low quality proteins by appropriate mixing of various other proteins is well known (Bressani, 1977). It could therefore be of interest to look at the consequences of replacing meat proteins with increasing amounts of collagen. In Figure 4, the chemical scores of different mixtures of meat, collagen, egg or pigskin are shown for male adults (FAO/WHO, 1973) children (FAO/WHO, 1973), and rats (Food and Nutritional Board, 1974, as cited by Steinke (1979)). The amino acid compositions used are from Table 3.

It is obvious, from this figure, that all essential and semiessential amino acids in collagen, except arginine, have lower chemical scores than in meat proteins. The thick line marks the limiting amino acid at different mixtures. Since the tryptophan content is zero in collagen this amino acid is limiting at higher concentrations of collagen in the mixtures. However, at lower collagen concentrations other amino acids are limiting. Thus the sulphur-containing amino acids are limiting (meat/collagen) for man and rats, and valine (meat/collagen) for children, but lysine (egg/collagen) for rats. The filled areas indicate the complementation effect obtained by mixing the two components compared to using **only** meat or egg proteins (at a constant protein level). The arrows indicate where maximum complementation is obtained in the mixture. At most, about 10-15 chemical score units can be gained for e.g. children (Fig. 4b) at this point. Thus it is apparent that collagen serves mainly as a non-specific nitrogen source and as an energy source when mixed with proteins from meat or eggs. Kofranyi & Jekat (1969) found that 50% of muscle protein could be replaced by gelatin without lowering the nutritive value for adults. Figure 4a shows that about 40% of the meat could be replaced by collagen and still a chemical score of 100 for adult man is obtained. As discussed in paper I it is evident that even if 25-40% of the protein in a meat product is collagen the protein nutritional value of that product would be adequate as the sole source of protein for weaning children.

In paper I supplementation of pigskin with methionine and tryptophan increased the biological value for rats from 32 to 43%. In Figure 4e it is shown that chemical scores of the other essential amino acid are quite close to each other, from about 40-60%. Supplementation must thus be performed with several amino acids to increase the quality considerably. This would also be true with regard to the needs of children (Fig. 4b).

Fig. 4. Chemical score (CS) for different mixtures. a) meat/collagen for adult man b) meat/collagen for children c) egg/collagen for rats d) meat/collagen for rats e) meat/pigskin for rats. arg = arginine; his = histidine; leu = leucine; iso = isoleucine; lys = lysine; met/cys = methionine plus cystine; phe/tyr = phenylalanine plus tyrosine; thr = threonine; try = tryptophan; val = valine. The thick line marks the limiting amino acid. The filled areas indicate the complementation effect obtained by mixing the two components compared to using only meat or egg proteins.





Collagen in meat products

Collagenous meat cuts are traditionally used in meat products. The collagen content varies between about 77% of the protein (pigskin) to about 2% (fillet) (Table 4). Using collagenous meat cuts surely influences e.g. firmness of sausage as shown in Figure 5; the more collagen, the firmer is the product. It has been stated that there is no evidence to suspect a hazard to the public health when using native collagen or regenerated collagen (sausage casings) in food (FDA 1981). From the nutritional point of view, collagen functions as a digestible protein partly utilized for complementation of the amino acid patterns in dietary protein, but mainly as a non-specific nitrogen and energy source. Replacing meat proteins with collagen, however, would also have consequences for other nutrients. Meat is a good source for several B-vitamins and minerals such as iron, zinc and selenium (Rogowski, 1980). Pigskin, however, has practically no B-vitamins (Lindner *et al.* 1965). The mineral content is probably also low since collagen itself does not bind iron, zinc or selenium, which is the case for meat proteins (Kühne, 1983; Robinson & Thomson, 1983).

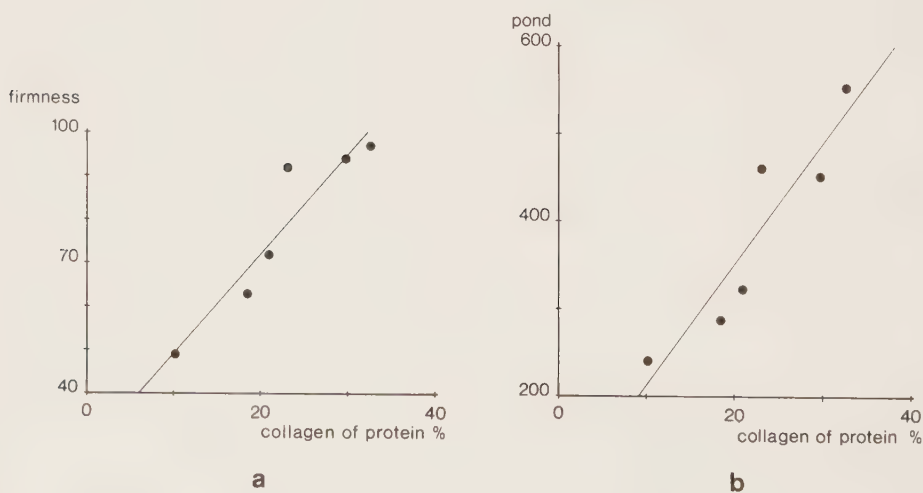


Fig. 5. Firmness of sausage with varying collagen content. Recipe based on sirloin, pigskin and backfat without pigskin. The products containing about 60 % water, 23 % fat and 9.5 to 13 % protein. a) Sensorial evaluation of uncooked sausage tested at room temperature; scale between zero (soft) and 100 (firm); a panel of 16 people. b) Instron Universal Testing Instrument using a conic penetrometer; 3 cm thick slices of sausage; temperature of 17°C; n=9.

Laser Reuterswärd, A., Andersson, L. & Rudérus, H. (1980). Nutritional value and technical properties of connective tissue in meat products. Report to the Swedish Farmers' Fund for Information and Development, May, Dossie 440-126/79.

Table 4. Protein and collagen contents of some raw materials and meat products.

Material	Total protein g/100g	Collagen of protein %	Reference
Beef cuts for sausage production	18	17	1)
Pork cuts for sausage production	16	10	1)
Pigskin	25	77	1)
Pork back fat with pigskin	8	67	1)
Pork back fat without pigskin	5	47	1)
Mechanically deboned meat:			
Beef	15	14	2)
Pork	16	11	2)
Standardized sausages	11	23	3)
Meat balls	15	11	3)
Fillet	21	2	4)

¹⁾ Analysis from Swedish Meat Research Institute

²⁾ Fuchs, G., Jansson, S. & Norberg, P. (1980). *Vår Föda*, 2, 50—63

³⁾ Fuchs & Kuivinen (1980)

⁴⁾ Lawrie (1979)

GENERAL CONCLUSIONS

1. Pigskin and tendon, containing about 75% and 95% collagen of the total protein, were highly digestible in rats. This was true for materials from young as well as old animals.
2. Pepsin, at pH 1.5, solubilized more than 80% of the protein in unheated pigskin from young animals as well as in calf and cow tendon.
3. *In vivo* digestion was usually more effective than *in vitro* solubilization of pigskin and tendon proteins.
4. The hydrochloric acid concentration *in vitro* and gastric acid secretion *in vivo* were both found to be important for the digestion of insoluble tendon collagen but not for gelatin and meat proteins.
5. In man, a collagenous fermented sausage, based on pigskin from scalded pigs, was digested to the same extent as a reference sausage based on meat.
6. Urinary hydroxyproline was excreted in free form when rats ingested hydroxyproline-rich diets. However, in man, the hydroxyproline was excreted mainly as peptides. The amounts excreted in the urine were only about 2% of the ingested hydroxyproline.
7. Undenatured collagen can be digested *in vitro* and *in vivo* without prior heat treatment and conversion to gelatin.
8. In rats, a linear decrease of Net Protein Utilization was found when the amounts of collagen increased in a mixture with meat proteins.
9. Collagen (gelatin) in mixed meat products could to some extent be utilized as complementation to meat proteins but could mainly be used as a non-specific source of nitrogen and energy.

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Effect of collagen content and heat treatment on protein digestibility and biological value of meat products

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Summary

The digestibility and biological value of meat mixed with various amounts of collagen were measured after heat treatments.

The M. biceps femoris muscle was used as the source of meat and pigskin as the source of collagen. The latter contained 78.9% collagen and digestibility both heated and raw was 95%.

The NPU of mixtures of meat and pigskin showed a relation with collagen content defined as $y = 82.8 - 0.6x$.

Supplementation of pigskin at a pigskin-meat mixture containing 50% collagen with methionine did not increase NPU.

The amount of collagen in untreated meat products is 15–30% and will be of negligible nutritional significance in a whole diet.

Introduction

The nutritive value of protein from muscle is known to be very high. The muscle proteins also suffer little or no damage when processed alone and denaturation does not affect protein quality (Bender, 1978). The proteins of the connective tissue, on the other hand, are generally considered to have little or no nutritive value. Furthermore collagen fibres are more resistant than most other protein fibres to attack by proteolytic enzymes (FDA, 1978) and meat collagen is considered to be only partly digestible. The low digestibility of collagen as well as of many unprocessed proteins, is considered to be due to naturally occurring crosslinks (Cheftel, 1977, 1979). With this background the Swedish National Food Administration has proposed legislation to limit the collagen content in meat products (Jansson, 1978).

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Different animal tissues and cuts of meat differ in content of connective tissue, depending on anatomic localization and physiological functions (Vognarova, Dvorak & Böhm, 1968). The proteins of the connective tissue are mainly collagen and elastin, but the amounts of elastin are very small both in muscle (Vognarova *et al.*, 1968) and in pigskin (Johns, 1977). Cuts with relatively high contents are usually in comminuted products such as sausages. A content of about 25% collagen, calculated on the crude protein is common in Sweden in comminuted products (Jansson, 1978). More than half of this collagen comes from pigskin and back fat with or without pigskin. The nutritive value of collagen in meat products is therefore of considerable interest.

The biological value (BV) of gelatin, which is solubilized collagen, is known to be very low (Bender, Miller & Tunnah, 1953). This is due to complete absence of tryptophan and low content of cystine (Eastoe, 1967). The total amount of essential amino acids is less than half of that in muscle proteins. The nutritive value of products rich in collagen has been investigated by some authors with rat assays. Studies on raw mixed meat products have shown decreasing NPU-values when the amount of collagen is increased (Dvorak, 1972; Bender & Zia, 1976). The PER-value decreases linearly with increasing collagen content in a mixture of raw beef round and partially defatted chopped beef (YuBang Lee *et al.*, 1978). The highest amount of collagen used in these studies was 50% of the total protein. In nitrogen balance experiments with adult humans however, Kofranyi & Jekat (1969) showed that 50% of muscle protein could be replaced with gelatin without lowering the nutritive value.

Thus data on digestibility and overall nutritional value of collagenous products are partly contradictory and difficult to evaluate. The data on NPU values so far reported are derived from carcass analysis, where BV and digestibility cannot be distinguished. The purpose of the present investigation was (a) to measure the protein digestibility of collagen of various particle sizes and after different heat treatments and (b) to study the protein quality of a highly collagenous product alone, mixed with various amounts of meat, and supplemented with amino acids.

Materials and methods

Mixtures of beef and pigskin were prepared in order to obtain samples with controlled contents of collagen and calculated on the basis of nitrogen. Heating parameters were chosen to be as close as possible to the conditions for sausage production and reheating respectively. The design of the experiments is shown in Table 1.

Preparation of meat samples

Muscles biceps femoris from beef and pigskin were obtained directly after the cutting process from a commercial slaughterhouse. The beef sample was carefully trimmed of excess fat and connective tissue and the pigskin was freed from

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Table 1. The nutritional values of mixtures of beef and pigskin heated under different conditions

Sample	Material	Mixture* (%)	Heating† conditions (°C)	TD‡	BV‡	NPU‡
1:1	Reference§			100.3 (0.9)	93.4 (1.4)	93.7 (1.9)
1:2	Beef/pigskin	100/0	raw	99.8 (0.4)	81.3 (4.2)	81.1 (3.9)
1:3	"	50/50	raw	99.2 (1.1)	66.3 (8.4)	65.8 (8.3)
1:4	"	100/0	74	100.3 (0.6)	78.1 (4.7)	78.4 (5.1)
1:5	"	50/50	74	99.3 (1.5)	58.8 (3.8)	58.4 (4.6)
1:6	"	100/0	95	99.0 (1.9)	83.2 (2.2)	82.3 (2.6)
1:7	"	50/50	95	99.2 (1.5)	64.9 (7.8)	64.3 (7.3)
2:1	Reference§			99.8 (0.8)	92.2 (2.2)	92.0 (2.5)
2:2	Beef/pigskin	100/0	74	99.4 (0.9)	78.4 (5.9)	77.9 (5.3)
2:3	"	80/20	74	100.4 (0.9)	68.5 (3.1)	68.8 (2.5)
2:4	"	60/40	74	100.6 (1.8)	64.1 (3.9)	64.5 (3.6)
2:5	"	40/60	74	99.3 (0.8)	56.1 (3.0)	55.7 (2.8)
2:6	"	20/80	74	97.6 (1.6)	44.6 (5.9)	43.5 (5.0)§§
2:7	"	0/100	74	95.3 (3.2)	32.9 (7.0)	31.2 (5.6)
3:1	Reference§			100.4 (0.7)	93.1 (1.9)	93.5 (1.9)
3:2	Pigskin + 0.40% Methionine		74	99.4 (0.7)	39.3 (7.0)	39.1 (7.3)**
3:3	Pigskin + 0.40% Methionine		74	98.1 (1.3)	42.8 (1.8)	42.0 (2.1)
3:4	Beef/pigskin + 0.40% Methionine	40/60	74	98.5 (1.5)	56.2 (2.9)	55.3 (2.7)
4:1	Reference§			101.5 (1.4)	90.8 (1.5)	92.1 (0.9)
4:2	Pigskin Ø < 0.8 mm		74	95.2 (2.2)	24.8 (8.3)	23.7 (8.2)
4:3	Pigskin Ø 0.8–2 mm		74	97.1 (0.8)	22.7 (3.9)	22.1 (3.7)

* Mixtures are based on nitrogen contents

† 30 min.

‡ ± standard deviation in parenthesis

§ Reference casein + 0.22% Methionine

§§ Calculated on four rats

** Calculated on three rats

meaty parts. The samples were finely cut in a bowl chopper, deep frozen and stored for 2 months. After thawing, portions of beef and pigskin were separately packed into plastic bags of saran lacquered polyester in 1-cm thick layers, heated for 30 min in a waterbath of $74^{\circ} \pm 1^{\circ}\text{C}$ and $95^{\circ} \pm 1^{\circ}\text{C}$, and then immediately chilled in ice-water. Samples were separately freeze-dried and then cut into a fine powder. The pigskin in experiment 4 was used directly after cutting, the particle diameter, measured with a sieve, being between 0.8 and 2 mm. In experiments 1–3 samples of pigskin were ground together with maize starch through a hammer mill with a filter of Ø 0.8 mm before being mixed into the diet for rat assay.

Chemical analyses

Chemical analyses was performed on raw samples before freeze-drying. Nitrogen (N) was determined by the method of Kjeldahl. Hydroxyproline (HPRO) was determined by the method of Stegeman (1958) modified by Weber (1973). The calculation of the content of collagen was made according to Prändl, Haas & Polke (1967):

$$\text{Collagen} = \text{HPRO} \times 7.1; \text{N}_{\text{Collagen}} = \text{Collagen}/5.55,$$

$$\% \text{ Collagen} = \frac{\text{Collagen}}{6.25 \times (\text{N}_{\text{total}} - \text{N}_{\text{collagen}}) + \text{Collagen}} \times 100$$

Amino acid analysis

The amino acid composition was determined by duplicate analysis of beef and pigskin heated to 74°C. Acid hydrolysis was employed and the equipment used was a Beckman 120 B amino acid analyzer. Cystine and methionine were determined after performic acid oxidation. Tryptophan was determined after alkaline hydrolysis according to Vangala and Menden (1970) and analysed according to Miller (1967).

Protein quality

The nutritive value of protein was evaluated with N-balance studies on growing rats. Male Sprague Dawley rats from Anticimex, Sweden, were placed individually in metabolic cages according to Shiller (1960) and Eggum (1973). After four days of acclimatization, urine and faeces were collected during a five day balance period with 5 rats in each group. The temperature was kept at 25°C and the relative humidity at 50–60%. The diet was composed according to Eggum (1973) of 15 g N/kg dry matter and with modified contents of vitamins and minerals according to Forsum, Hambræus & Siddiqi (1973). Maize starch was used as source of carbohydrate. ANRC Reference Protein High Nitrogen Casein (Sheffield Chem. USA) was used as reference protein. Amino acid supplementation was carried out with L-Methionine (Biochemische Zwecke, Merck) and L-Tryptophan (chromatographically homogenous, BDH). Amino acid supplementations are expressed as % of total diet prepared to contain 1.5% nitrogen, corresponding to 9.4% crude protein. Supplementation design is shown in Table 1.

The N contents of the feed, urine and faeces were analyzed by the method of Kjeldahl. Values for metabolic and endogenous N used in the calculations of TD and BV were obtained from experiments with 0.6% N from fat extracted, lyophilized whole egg as source of protein.

Results and discussion

Chemical composition

The beef contained 3.39 gN and 0.15 g HPRO per 100 g. The corresponding figures for pigskin were 4.91 and 3.10 respectively. So the collagen content in beef and pigskin were 5.1 and 78.9 per cent of the total protein respectively.

Digestibility

Data from experiment 1, Table 1, indicate that there are no significant differences in digestibility between raw and heated samples. The digestibilities of mixtures of equal parts of beef and pigskin, which correspond to 42% collagen, were more than 99% and comparable to the values for beef with only 5.1% collagen content. The lowest value of digestibility, 95%, was obtained for pigskin alone (2:7, 4:2, Table 1). In experiments 1–3 the pigskin was milled into particles with a diameter of less than 0.8 mm. Experiment 4 (4:2, 4:3 Table 1) showed that the digestibility was more than 95% even with particles of about 2 mm diam., as in most comminuted products.

Our findings are in agreement with earlier publications, all indicating that collagenous products are well digested. Mitchell & Carman (1926) considered the protein digestibility to be the same in cheap and expensive cuts of meat. Happich *et al.* (1975) showed that partially defatted chopped beef with about 50% collagen, heated to a maximum of 49°C, had a digestibility of 88%. Both raw and cooked pigs ears with high amounts of collagen were found to be 95% digestible (Vaughn, Wallace & Forster 1979). Brüggeman *et al.* (1964) refer to *in vitro* studies on raw and cooked pigskin where the digestibilities were 97.2 and 97.8% respectively. To our knowledge no investigation has been published that clearly shows low digestibility of collagen *in vivo*.

Vognarova *et al.* (1968), suggested that the content of solubilized collagen in heated muscle could be an indication of digestibility. Heating of the samples in this investigation has probably partly denatured the collagen. Johns & Courts (1977) refer to extraction studies of pigskin which gave a gelatin yield of only 4.5% after 2 hrs at 80°C of neutral pH. Hill (1966) found that in young as well as old animals less than 20% of the collagen in muscle was solubilized after one hours heating at 77°C. The heating used during sausage production and mild reheating evidently does not solubilize collagen to any larger extent. Furthermore in our study the digestibility was very high even in the raw product.

Apparently the opinion that collagen has a low digestibility is based not only on data concerning the low solubility of collagen after heating, but also on the fact that some proteolytic enzymes are ineffective in degrading collagen *in vitro*. Thus Neumann & Tytell (1950) did show that trypsin, chymotrypsin and papain were practically ineffective on collagen from different sources. However, from several early investigations, reviewed by Brüggeman *et al.* (1964), it seems that

collagen might be broken down in the stomach before passing into the intestine. These investigations show that pepsin and stomach acid do hydrolyze native collagen.

Biological value and NPU

Since the digestibility of collagen in pigskin is so high, NPU values mainly reflect the BV. Thus the differences of NPU are mainly due to variations in the amino acid composition of different collagenous products. Results of BV and NPU are shown in Table 1. Data from experiment 2 (Fig. 1) show a direct relationship between NPU and content of collagen, with the regression equation defined as $y = 82.8 - 0.6x$ and a correlation coefficient of 0.99. For pigskin, with 78.9% collagen, a NPU value of 36 can be deduced from the equation. Linear extrapolation to 100% collagen could then be related to a NPU of 23. This is in accordance with the concept that BV approaches 20 when the tryptophan content of a protein, limited by this amino acid, is decreasing towards zero (Bender, 1973) as in collagen. Present results are also in accordance with those of Bender & Zia (1976) who showed a NPU value of 69 for shin with 23.6% collagen and a NPU value of 82 for fillet with 2.5% collagen. NPU values of 69 and 81 respectively can be deduced from the equation in Fig. 1 for these contents of collagen. Corresponding NPU values deduced from the study by Dvorak (1972) are 65 and 83 respectively.

Amino acid supplementation

Supplementation of pigskin with methionine (3:2 Table 1) and methionine plus tryptophan (3:3 Table 1) did not significantly increase the NPU-value (compared to 2:7, Table 1). The reason for this is probably that the isoleucine

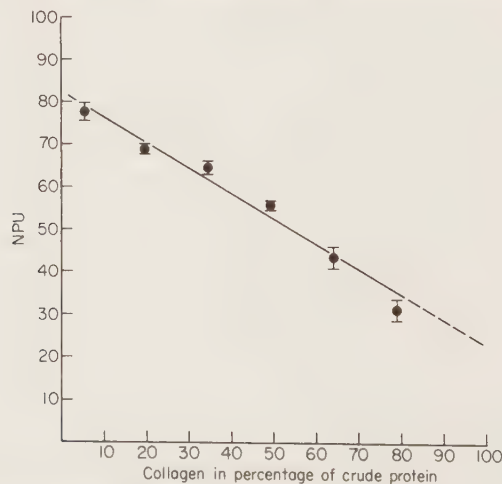


Figure 1. Relationship between NPU-value and collagen content in mixtures of M. biceps femoris from beef and pigskin (mean values and s.e. mean).

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content of pigskin is also low, which immediately makes this amino acid limiting after the supplementation. The chemical score values from Table 2 indicate a value of about 30 for both tryptophan, methionine plus cystine and isoleucine. Supplementing a 50% collagen mixture with methionine (3:4, Table 1) did not increase the NPU. Table 2 shows that chemical scores for isoleucine, valine, methionine plus cystine, leucine, threonine and tryptophan of such a mixture are all between 57–66%. Thus it is obvious that supplementation must be performed with several amino acids in addition to methionine and tryptophan to increase the quality of a mixture of 50% collagen to the same level as muscle protein. At levels of about 25% of collagen it seems however enough to supplement just one amino acid. Thus Bender and Zia (1976) supplemented shin with methionine, increasing the NPU-value to a level above that of fillet.

Table 2. Essential amino acids and chemical scores for beef and pigskin

	Beef (g/16gN)	Pigskin (g/16gN)	Pigskin (Chemical score)*	Beef/pigskin 40/60 (Chemical score)†
Isoleucine	3.7	1.3	33	57
Leucine	6.6	2.9	41	63
Lysine	7.1	3.2	58	87
Methionine + cystine	3.6	1.2	34	62
Phenylalanine + tyrosine	6.0	2.9	48	69
Threonine	3.9	1.8	45	66
Tryptophan	1.2	0.3	30	66
Valine	4.0	2.1	42	57

*Calculated from figures from column 2 and data from FAO/WHO (1973).

†Calculated from figures from column 1 and 2 and data from FAO/WHO (1973).

The discussions above concerning supplementation are only relevant for growing children. In the study of Kofranyi & Jekat (1969) 50% of muscle protein could be replaced with gelatin without lowering the nutritive value for adult humans. The obvious explanation for this is that the need for essential amino acids is much lower for adults than for growing children (FAO/WHO, 1973). Comparison of essential amino acid patterns (Table 2) with estimated needs for adults (FAO/WAO, 1973) indicate that methionine plus cystine is limiting in the mixture containing 50% collagen, and that the content of these amino acids is only slightly below the need.

Conclusions

Collagen from pigskin has been shown to have a digestibility of about 95% in rat assay, when the particle size of collagen is about 2 mm as in comminuted meat

products. This was true both for raw and heated samples, and probably applies also to other collagenous materials used in food.

BV and thus NPU decreased linearly with increasing collagen content. The regression equation between NPU and % of collagen of the crude protein is defined as $y = 82.8 - 0.6x$. Supplementing a 50% collagen mixture with methionine did not increase the NPU value. In order to obtain an increase, supplementation must be performed with several amino acids. The acceptable NPU level in mixed meat products is a matter for discussion. For weaning foods PAG (1971) recommends a NPU value of not less than 60 and preferably above 65. This corresponds to a collagen content of 38% and 30% respectively in the present study. PER value of 2.5 proposed for interim legislation by USDA 1976 corresponds to 28.5% collagen in the investigation of YuBang Lee *et al.* (1978). From these data it is evident that even if 25–40% of the protein in a meat product is collagen the protein nutritional value of that product would be adequate as the sole source of protein even for weaning children.

In a mixed diet the practical levels of collagen in industrial meat products (15–30%) will be of negligible nutritional significance.

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Solubilization of pigskin and bovine tendon after pepsin and pancreatin treatment. Effect of incubation conditions and age of animal

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SUMMARY

Solubilization of pigskin, bovine tendon, insoluble collagen (bovine Achilles tendon); gelatin and meat was studied using pepsin and pancreatin, at 37°C for up to 4 hr. In some cases precipitation of remaining protein with trichloroacetic acid (TCA) was used. The parameters studied were: pH during the pepsin/HCl incubation, the effect of pancreatin either alone or after pepsin action, the effects of animal age and importance of prior heat treatment of the samples. Results showed that pepsin at pH 1.5 solubilized 79-88% of the protein in unheated young pigskin as well as in calf and cow tendon. The young pigskin sample was more soluble in pepsin/pancreatin/TCA than the older one, 94 and 75%, respectively. No age effect was obtained for tendons, which were all more than 83% soluble after pepsin/pancreatin treatment. Pancreatin alone solubilized about 15% of young and old pigskin proteins. The solubility after pepsin and TCA precipitation was less than 10% for all pigskin and tendon samples but pepsin and pancreatin treatment resulted in TCA-soluble peptides.

Heated samples had a bigger particle size than the corresponding unheated samples. The overall effect of heat treatment was statistically insignificant when considering both pepsin and pepsin/pancreatin/TCA solubilities.

Solubilization of unheated old pigskin protein and insoluble collagen was highly influenced by pH during the pepsin incubation. Already at pH 2.0-2.5 the solubility decreased reaching about 50% at pH 3. Further treatment with pancreatin only slightly increased the solubility values. Gelatin and meat samples were highly solubilized by pepsin and pancreatin and not precipitated by TCA. The prominent pH effect was not observed for these samples.

The solubilization data of unheated pigskin and raw tendon indicate that the digestibility of collagen could be high also *in vivo*. However, pH during the pepsin incubation seems to be critical for degradation of insoluble collagen.

INTRODUCTION

With the exception of collagenases, which are unique in their ability to degrade native collagen to small dialysable peptides, collagen is extremely resistant to proteolytic enzymes. Enzymatic degradation of native collagen has been studied by several authors with different aims and reviewed extensively in connection with the leather industry (Gustavson, 1956), gelatin manufacture (Balian & Bowes, 1977; Johns & Courts, 1977), biochemistry and medicine (Mandl, 1961; Weiss, 1976; Bailey & Etherington, 1980) and tenderness of meat (Sørensen, 1976; Sørensen, 1981). In the literature, data on the digestibility of collagenous food

products somewhat conflict and have been under discussion since at least 1910 (Baumstark & Cohnheim, 1910). Studies performed are partially reviewed by Brüggeman *et al.* (1964), Loewit (1967; 1970) and Ashgar & Henrickson (1982). A common opinion is that native collagen resists digestion (Cheftel, 1977; Prandl, 1980; Kies, 1981; Ashgar & Henrickson 1982). In order to become digestible, wet heat treatment of the collagen is regarded necessary resulting in the formation of gelatin (Rogowski, 1980). According to this view denaturation by cooking makes collagen susceptible to enzymes (Paul, 1972; Ashgar & Henrickson, 1982) and thus digestible (Cheftel, 1979).

Only a few *in vitro* studies on enzymatic degradation of highly collagenous materials using pepsin and hydrochloric acid (HCl) at 37°C have been performed. Neuman & Tytell (1950) treated Achilles tendon and solubilized about 95%. Franke (1964) found that both raw and heated pigskin were about 97% 'digestible'. On the other hand Loewit (1967), treating samples with pepsin/HCl and pancreatin, reported the 'digestibilities' of raw pigskin and tendon to be only 38% and 48%, respectively. After cooking, the 'digestibilities' increased to 100% and 79%, respectively. Harkness, Harkness & Venn (1978) treated rat tail tendon and bovine tendon with pepsin/HCl and achieved a solubilization of 97% and 37%, respectively. In these investigations solubility was used as the measurement of digestibility. However, it should be noted that the collagen helix can be solubilized intact after disruption of the telopeptide region by pepsin (Weiss, 1976). Thus, the solubility of collagen does not necessarily indicate the extent of degradation.

The *in vitro* studies mentioned above have all been performed on collagenous materials from animals of unspecified ages. However, it is well known that insoluble collagen from a growing animal shows an increasing resistance to solubilizing agents, such as pepsin, with age (Weiss, 1976; Sims & Bailey, 1981).

In the present investigation solubilization of pigskin and bovine tendon protein after pepsin and pancreatin treatment was studied. Conditions in the stomach and small intestine were simulated. The influence of some parameters were of special interest: a) pH during incubation with pepsin b) the extent of trichloroacetic acid precipitation after solubilization c) age of animal d) prior heating of pigskin and tendon.

MATERIALS AND METHODS

Preparation of samples

Pigskins

Skins were removed immediately after slaughter from a 4 month old pig and a 5 year old sow. The pigskins were manually shaved with a razor. Another skin was taken from a 6 month old pig which had been scalded according to slaughterhouse procedures. All the pigskins were freed of visible fat and meaty parts. Pigskin samples were finely minced in a Moulinex blender. Half of the samples were vacuum packed in thicknesses of less than 1 cm, heated in a waterbath at $74^{\circ}\pm 1^{\circ}\text{C}$ for 30 min and immediately chilled. The heated samples were finely reminced in a Moulinex blender. All samples were freeze-dried. The unheated samples were then minced in a knife homogenizer (Janke & Kunkel) and the heated samples in a Moulinex blender.

Tendons

Achilles tendons were collected from forty 7 month old calves and from forty cows with an average age of 6.6 years (range 4 to 12.5). The calf tendons were collected 24 hours and the cow tendons 1-6 days after slaughter. Samples were freed of visible fat and meaty parts and frozen for not more than 2 months. After thawing, the tendons were cut into smaller pieces and mixed into one calf and one cow sample. The wet calf and cow samples were ground and passed through a screen of 3 mm before being immediately chilled. The temperature of the calf sample reached 32°C and the cow sample 44°C during grinding. Half of the cow sample was packed and heated as described for the pigskin samples above, and minced in a Moulinex blender. The unheated calf and cow samples as well as the heated cow sample were freeze-dried. Parts of the samples were homogenized in a hammer mill.

Meat

Meat of low collagen content was taken from the top round (*M. semimembranosus* and *M. adductor*), minced in a Moulinex blender, frozen, freeze-dried and reminced in a Moulinex blender to a fine powder. Shin, with relatively high amounts of collagen, was freed of visible fat and meaty parts, minced in a Moulinex blender and frozen. The sample was thawed and used wet as the collagen fibers were difficult to cut after freeze-drying.

Commercial samples

Insoluble collagen (bovine Achilles tendon) prepared according to Einbinder & Schubert (1951) and gelatin from swine skin (175 Bloom) were purchased from Sigma, Chem. Comp., USA.

Determination of particle size

All freeze-dried samples were surveyed under a low magnification microscope. The particle diameter was estimated and particles were photographed.

Enzymes

Porcine pepsin NF (Merck) and pancreatin from porcine pancreas 4 NF (Sigma Chem. Comp., USA) were used. The enzymes were routinely checked for hydroxyproline-blank. The collagen content was 23.5% for pepsin and 3.9% for pancreatin expressed as collagenous nitrogen versus total nitrogen.

Pepsin incubation

Samples with 0.08 g nitrogen were mixed with 44 ml of 0.03 M HCl and the pH was measured before and after incubation. The initial pH-values were 1.35-1.53 for all pigskin, tendon and gelatin and insoluble collagen samples and 1.62 for the meat samples. The pH-values after incubation generally increased by 0.05 but in a few cases by 0.30 units. One ml of pepsin solution (50 mg) was added and incubation was performed in a shaking waterbath at 37°C for 2hr. Samples were then chilled, the pH adjusted to 7.0 with 4 N NaOH and of water added up to 50 ml. Suction filtering through a glass filter with a porosity of 15-40 μ m was used. An aliquot of the filtrate was precipitated with trichloroacetic acid (TCA) 1:1, usually to a final concentration of 25% w/v. After standing at +5°C overnight, samples were filtered through an OOH filter to obtain a clear solution.

Pepsin/pancreatin incubation

After the pepsin incubation, the pH was adjusted to 6.8 with 4 N NaOH and up to 50 ml of water was added up to 50 ml. Thereafter 25 ml of 0.1 M sodium-phosphate buffer pH 6.8 was added as well as 50 mg of pancreatin. Incubation was performed at 37°C for 2hr, samples were chilled in ice-water and suction filtered as above. An aliquot of the solution was treated with TCA 1:1 usually to a final concentration of 6.5% w/v, left standing overnight at +5°C and filtered as above.

Different incubation conditions

Young and old unheated pigskin samples were also studied with some other incubation conditions: a) 0.03 M HCl for 2hr followed by 0.1 M sodiumphosphate buffer for 2hr, b) pancreatin for 2hr or 4hr, or c) 0.03 M HCl for 2hr followed by pancreatin for 2hr. The TCA used was of a final concentration of 25% w/v. The effect of the HCl concentration (0.03 M - 0.0001 M) was also studied during incubation with pepsin for 2hr followed by pancreatin incubation for 2hr and precipitation with TCA (final concentration 25% w/v). This was performed on old unheated pigskin and insoluble collagen, gelatin and non-collagenous meat samples.

Chemical analyses

Analyses were performed on each fraction for nitrogen and in some cases for hydroxyproline. Corrections were made for blank values obtained with enzymes only under the same conditions. All incubations were performed on five parallel samples unless otherwise indicated.

Nitrogen was determined on powdered samples and on solutions using the Kjeldahl method. Hydroxyproline was determined using a Technicon Auto-Analyzer on duplicate samples, according to Stegemann (1958) as modified by Weber (1973). Calculations of collagen and protein contents were made as described previously (Laser Reuterswärd *et al.* 1982). Water content on all powdered samples was determined by drying overnight at 104°C.

Statistical evaluation

Means and standard deviations were calculated and analyzed by Student's t-test for small samples and unknown variances assumed equal (Bailey, 1981).

RESULTS

Composition and structure

The water content of the freeze-dried samples varied between 1.1 and 7.8%. Protein and collagen contents are shown in Table 1. The variations in protein content were due mainly to the varying amounts of fat.

Some representative microphotographs are shown in Fig. 1. Unheated pigskin samples had a more porous, crystalline-like, tight structure than the corresponding heated samples, which were aggregated and transparent. The particle sizes of unheated young and old pigskin samples were the same. The particles of the corresponding heated samples were much larger. The size of pigskin samples were

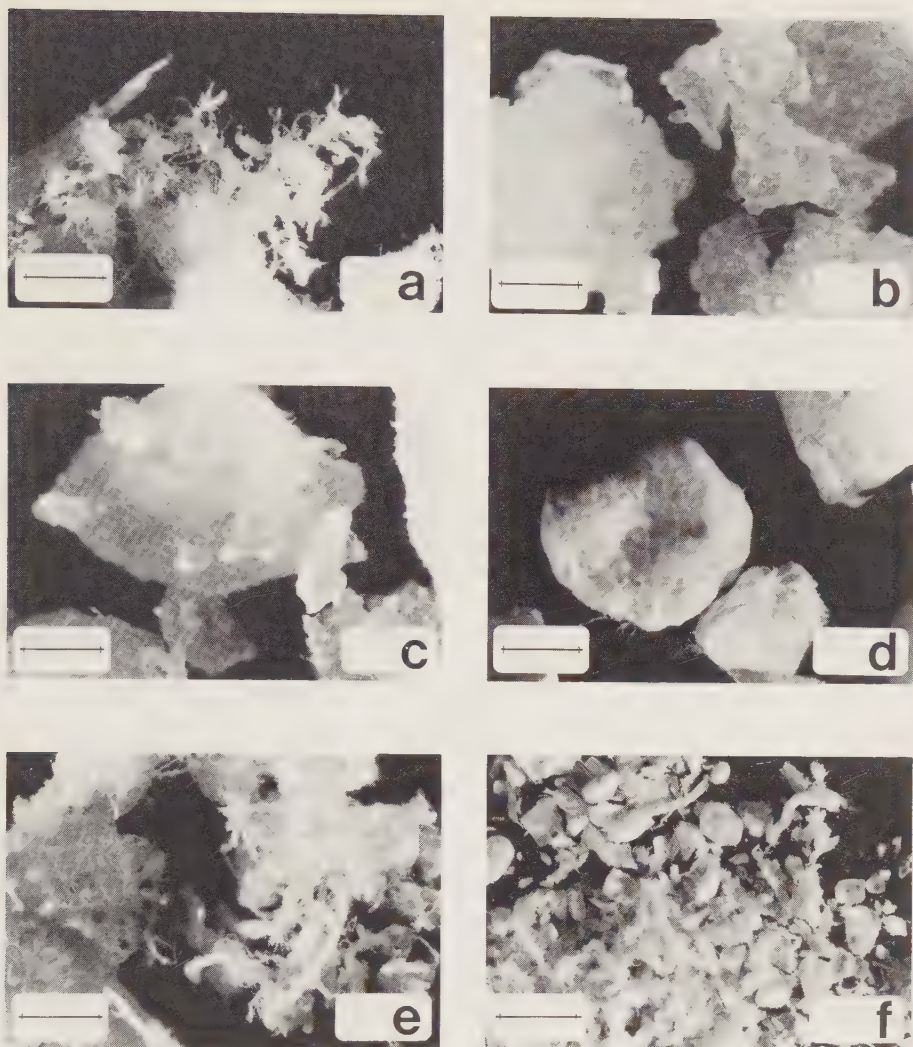


Fig. 1. Microscope photographs of pigskin and bovine Achilles tendon samples, freeze-dried and minced: homogenized (Janke & Kunkel) = JK, Moulinex blender = MB and hammer-milled = HM. Heat treatment was performed at 74°C for 30 min. The scale shown on the photographs is equivalent to 1 mm.

- a) young (4mo) unheated pigskin, JK*
- b) young (4mo) heated pigskin, MB*
- c) young (6mo) scalded pigskin, MB*
- d) cow, (6.6yr), MB*
- e) cow, (6.6yr), HM*
- f) cow, (6.6yr), heated, HM*

1-3 mm for the old heated pigskin, 3-4 mm for the young heated pigskin and 2-4 mm for both the young scalded and scalded/heated pigskins. Unheated, non hammer-milled tendon samples looked spongy, the calf sample had a particle size of 2-3.5 mm and the cow sample 1-2.5 mm. Unheated, hammer-milled calf and cow tendon samples were porous and crystalline-like (Fig. 1e) and the heated hammer-milled cow tendon sample showed more tight particles (Fig. 1f). Insoluble collagen was porous but had smaller particles than pigskin and tendon samples. Gelatin and meat samples had extremely small particles, the gelatin being transparent.

TCA-solubility

TCA is normally used for precipitating proteins from peptides (Oser, 1965). However, at a 5% TCA concentration collagen is still soluble and possible to separate from other proteins (Harkness *et al.*, 1978). Therefore, final concentrations of zero to 25% w/v of TCA were tested using samples from meat and old unheated pigskin.

After treatment with pepsin/HCl at pH 1.5 the concentration of TCA weakly influenced the precipitation of meat proteins with a linear regression equation of $y = -9.53x + 0.134$ and a linear regression coefficient significantly different from zero ($P < 0.01$). For pigskin, TCA concentrations equal to or more than 15%, gave significantly lower solubilities ($P < 0.001$) than the constant level found when using 0, 5 and 10% TCA. After pepsin/pancreatin treatment, six concentrations of TCA ranging from zero to 25% were tested, but the linear regression coefficient was not significantly different from zero ($P > 0.05$) for either the meat or the pigskin samples. For precipitating samples treated with pepsin/HCl at pH 1.5 followed by pancreatin, a TCA concentration of 6.5% w/v was chosen but for all other samples a concentration of 25% w/v TCA was used.

The TCA-solubilities after only pepsin incubation were less than 10% for all the pigskin and tendon samples, likewise gelatin, and independent of age and heat treatment (Table 1).

The TCA-solubilities after pepsin/pancreatin treatment (Table 1) showed no significant difference from values without TCA-precipitation, when all values were tested with a paired t-test.

Effect of age and heat on solubilization

In Table 1 the results of solubilization with pepsin and pepsin/pancreatin are shown for pigskin, tendon, insoluble collagen, gelatin and meat samples.

Since the particle size of the unheated pigskins were the same, this factor could not influence the comparison of various samples from animals of different ages. The young unheated pigskin sample was significantly ($P < 0.001$) more solubilized than the old unheated pigskin sample. This was true both for solubilities after pepsin treatment alone and after pepsin/pancreatin/TCA treatment. In spite of the somewhat bigger particle size of the young heated pigskin sample this was significantly ($P < 0.001$) more soluble after pepsin, as well as after pepsin/pancreatin/TCA than the old heated one. Thus age had an influence on the solubilization of both unheated and heated pigskin samples.

The overall effect of heat treatment was insignificant according to the paired t-test, when including pepsin and pepsin/pancreatin/TCA solubilities. However,

Table 1. Composition and solubilization of pigskin, bovine Achilles tendon, insoluble collagen, gelatin and meat samples. Solubilities are expressed as a % of soluble nitrogen of initial nitrogen. Incubation was performed with pepsin at pH 1.5 for 2hr; or pepsin at pH 1.5 for 2hr followed by pancreatin at pH 6.8 for 2hr at 37°C. Precipitation was performed with 25 % w/v or 6.5 % w/v of Trichloroacetic acid (TCA). Heat treatment was performed at 74°C for 30 min. n=5, standard deviations within brackets. HM=hammer-milled.

Sample	Protein/ dry weight %	Collagen/ protein %	SOLUBILITY %			
			Pepsin	Pepsin/ TCA	Pepsin/ pancreatin	Pepsin/ pancreatin/ TCA
<u>Pigskin</u>						
Young, 4 mo unheated	47.4	71.8	84.1 (3.9)	6.8 (0.6)	92.0 (3.5)	94.2 (4.0)
heated	52.1	71.7	86.3 (1.7)	5.9 (0.6)	98.4 (3.0)	97.0 (2.1)
Young, 6 mo scalded	58.1	71.3	78.8 (2.3)	4.7 (0.4)	82.6 (4.0)	82.6 (3.5)
scalded/heated	63.5	73.7	88.6 (4.0)	6.2 (1.0)	94.5 (1.7) ¹⁾	96.5 (2.9) ¹⁾
Old, 5 yr unheated	70.2	76.1	72.8 (1.8)	5.7 (0.8)	76.5 (2.3)	75.2 (3.5)
heated	70.8	77.6	60.8 (1.4)	3.6 (1.1)	79.4 (3.2)	81.4 (1.6)
<u>Tendon</u>						
Calf, 7 mo unheated, not HM	93.5	92.5	78.9 (0.8)	5.2 (0.5)	86.6 (3.3)	86.8 (1.7)
Calf, 7 mo unheated, HM	93.5	92.5	86.0 (3.1)	6.5 (0.6)	90.4 (1.6)	92.4 (8.9)
Cow, 6.6 yr unheated, not HM	99.7	96.7	81.8 (3.8)	4.7 (1.3)	83.8 (3.5)	83.5 (3.7)
Cow, 6.6 yr unheated, HM	99.7	96.7	87.5 (5.7)	10.3 (0.7)	92.7 (1.7)	91.9 (2.4)
Cow, 6.6 yr heated, HM	97.2	94.8	88.0 (2.2)	5.6 (1.0)	91.9 (2.1)	92.8 (0.9)
Insoluble collagen	102.2	96.4	91.4 (2.2)	2.4 (0.5)	92.2 (3.6)	92.5 (3.0)
Gelatin	99.8	92.0	93.0 (0.9)	8.0 (0.7)	101.0 (2.1)	100.0 (2.3)
Meat, top round	94.2	1.8	94.9 (1.5)	72.4 (1.4)	100.6 (2.0)	100.5 (1.7)
Meat, shin	72.4	16.5	92.2 (2.3)	56.7 (2.3)	91.5 (1.8)	91.8 (2.4)

¹⁾ n=3

all heated samples had a bigger particle size than the corresponding unheated samples. This could have counteracted a heat effect. For the young pigskin sample no significant difference was found after heat treatment either for pepsin solubility or pepsin/pancreatin/TCA solubility. For the old sample a significantly lower ($P<0.001$) value for the heated sample was obtained after pepsin treatment alone but a significantly higher ($P<0.01$) value for the pepsin/pancreatin/TCA solubility. The young scalded sample had the same particle size as the scalded/heated sample and in this case a significant ($P<0.01$) increase in solubility was obtained for both the pepsin solubility and the pepsin/pancreatin/TCA solubility due to heat treatment. The young scalded sample showed significantly ($P<0.05$) lower solubility than the young unheated (unscalded) one, both as pepsin and pepsin/pancreatin/TCA solubility. This was probably due to the larger particles in the scalded sample.

For the tendon samples hammer-milling significantly increased the solubility ($P<0.01$) for the calf and cow samples, both after pepsin alone and after pepsin/pancreatin/TCA treatments. The particle size thus influenced the solubility of tendon samples.

For the tendon samples no significant age effect was found either for the hammer-milled calf and cow samples, or for the non-hammer-milled calf and cow samples. This was true for both the pepsin and the pepsin/pancreatin/TCA solubilities.

Insoluble collagen, with its smaller particles, showed a significantly higher ($P<0.05$) solubility in pepsin than the hammer-milled calf sample, but an insignificant difference compared to the hammer-milled cow tendon sample. The pepsin/pancreatin/TCA solubility was, for insoluble collagen, not significantly different from either the hammer-milled calf or the cow samples.

No significant difference in solubility (pepsin and pepsin/pancreatin/TCA) was found after heat treatment of the cow sample compared to the unheated hammer-milled one. Significantly higher values (pepsin and pepsin/pancreatin/TCA) were found when comparing the heated cow sample to the non-hammer-milled sample ($P<0.05$).

After pepsin treatment, the solubility of gelatin was not significantly different from that of insoluble collagen, but after treatment with pepsin/pancreatin/TCA a significantly higher ($P<0.01$) solubility was obtained for gelatin than for insoluble collagen.

The collagenous meat sample had a pepsin/pancreatin/TCA value significantly lower ($P<0.01$) than the completely solubilized meat sample.

The effect of incubation conditions

Table 2 shows the impact of different combinations of HCl, buffer and enzyme incubations on the solubilities of unheated pigskins from young and old animals. Generally the older pigskin was much less solubilized than the young sample, except after the pancreatin treatment. The HCl/buffer solubilized only a small amount of nitrogen. Pancreatin treatment, for 2hr or 4hr, solubilized even less nitrogen. Pretreatment with HCl gave a significant ($P<0.01$) increase in pancreatin solubilization for both the young and the old samples. TCA did not precipitate nitrogen solubilized by pancreatin treatment.

The solubility of especially the old pigskin sample was much greater after incubation in HCl/pepsin than in HCl and pancreatin alone. Pancreatin degraded all the HCl/pepsin solubilized nitrogen to TCA-soluble nitrogen, both in young and old samples. The hydroxyproline-solubility showed the same pattern as the nitrogen-solubility.

Fig. 2 shows the effect of pH during incubation with pepsin alone or with pepsin/pancreatin/TCA treatment on the old unheated pigskin, insoluble collagen, gelatin and non-collagenous meat samples. At a critical pH the solubilities started decreasing with different slopes. For the old unheated pigskin and insoluble collagen this critical point, for the pepsin as well as for the pepsin/pancreatin/TCA solubilities, was in the range of pH 2.0-2.5. At pH 3.0 the solubilities were about 50%. For the gelatin sample a small decrease started around pH 3.0, but at pH 5.0 the solubility was still 92%. For the meat sample the critical pH was around 2.5, the solubility decreasing to 80% at pH 4.0.

Table 2. Solubilization of unscalded, unheated young and old pigskins. Samples were incubated in hydrochloric acid (HCl) at pH 1.5, sodium-phosphate-buffer/pH 6.8, pepsin and pancreatin at 37°C. Solubilities are expressed as % soluble nitrogen of the initial nitrogen and % soluble hydroxyproline (HPRO) of the initial HPRO. Trichloroacetic acid (TCA) at 25 % w/v was used (TCA-solubility).

In vitro design				Solubility %				TCA-solubility %			
HCl	HCl/ Pepsin	Buffer	Buffer/ Pancreatin	Young		Old		Young		Old	
				Nitrogen ¹⁾	HPRO ²⁾	Nitrogen	HPRO	Nitrogen	HPRO	Nitrogen	HPRO
2hr	—	2hr	—	29.7 (1.7)	20.2	10.3 (1.2)	5.2	4.0 ³⁾	—	2.7 ³⁾	—
—	—	—	2hr	12.8 (0.8)	—	15.0 (0.8)	—	11.8 (1.8)	—	12.5 (0.7)	—
—	—	—	4hr	15.3 (0.4)	—	16.1 (1.4)	—	—	—	—	—
2hr	—	—	2hr	62.5 (3.0)	—	28.3 (2.7)	—	60.6 (4.0)	64.2	28.1 (4.7)	26.6
—	2hr	—	—	84.1 (3.9)	86.3	72.8 (1.8)	64.2	6.8 (3.9)	2.8	5.7 (0.8)	2.8
—	2hr	—	2hr	92.0 (3.5)	91.7	76.5 (2.3)	69.7	94.2 (4.0)	94.6	75.2 (3.5)	71.8

¹⁾ Nitrogen, *n* = 5 from incubation to Kjeldahl analysis, standard deviations within brackets

²⁾ HPRO is duplicated on one sample with a nitrogen value close to average

³⁾ *n* = 1

DISCUSSION

Preparation of samples

The samples used were prepared by grinding wet material and freeze-drying and in some cases by hammer-milling the freeze-dried samples. The temperature of the wet tendons after grinding was less than 44°C. Gustavsson (1956) reported that grinding of native collagen could result in somewhat increased solubility after trypsin treatment. However, the denaturation temperature is about 65°C for wet collagen fibers (Bailey, 1968) and much higher for dried fibers (Finch & Ledward, 1972). Hammer-milling of the tendon samples resulted in smaller particles (Fig. 1d, e) and significantly increased solubilities. This is in accordance with Banga & Balo (1965) who also obtained a higher solubility of air-dried hammer-milled Achilles tendon than of fibres treated with collagen mucoproteinase. This was explained as being caused by physical disintegration.

Freeze-drying is a procedure normally used when studying the chemical crosslinks of collagen (Light & Bailey, 1980). However, water undoubtedly has a fundamental role in maintaining the native collagen structure and when dehydrated collagen loses its regular structure (Privalov, 1982). This also occurs after freeze-drying (Bailey, Robins & Balian, 1974). Adding water to dehydrated collagen again restores the structure (Privalov & Tiktopulo, 1970). It is unknown, however, if full structural restoration occurred during the relatively short incubation time used in the present study. The freeze-drying would not have overestimated the solubility values since freeze-drying is known to result in decreased solubility of fibers (Dutson, 1976).

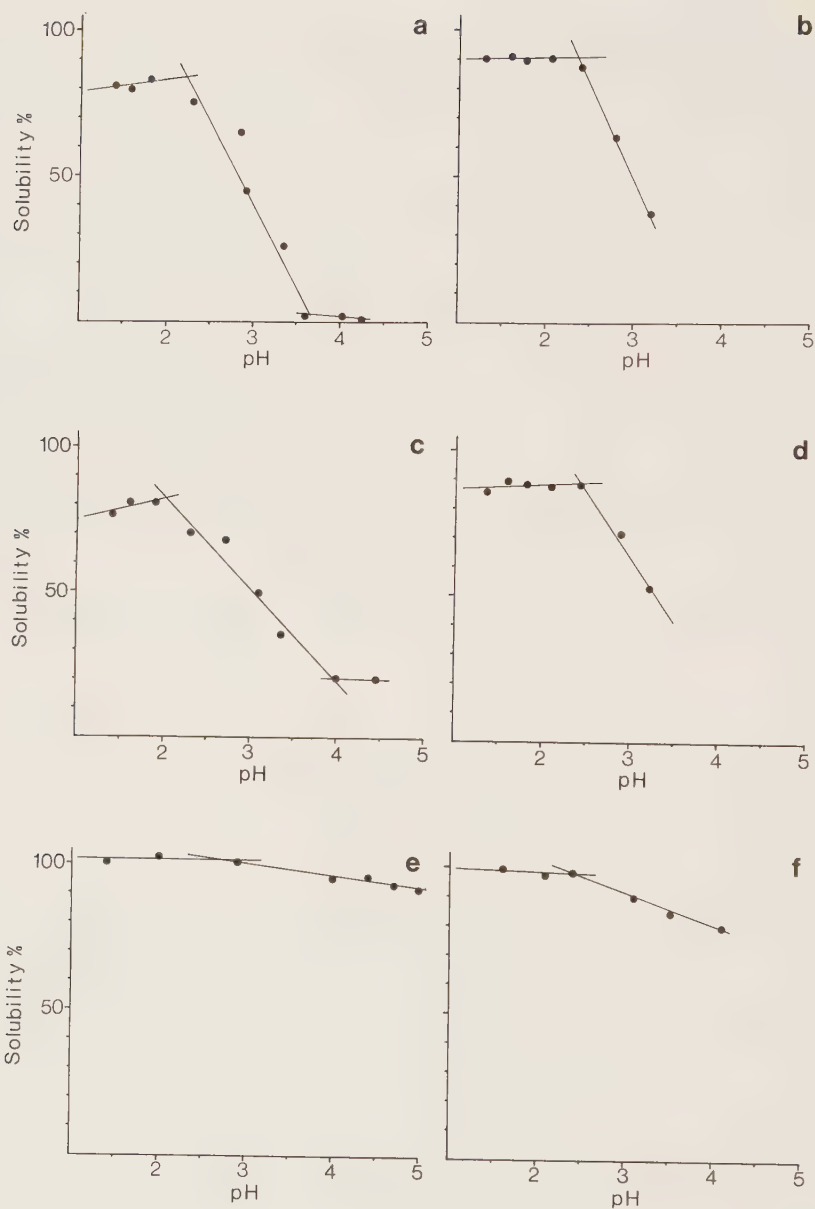


Fig. 2. Nitrogen solubility, as a % of initial nitrogen, after incubation for 2hr at 37°C with pepsin and hydrochloric acid at different pH, in c), d), e), f) followed by incubation for 2hr at 37°C with pancreatin, pH 6.8. The pancreatin digests were precipitated with final concentration of 25 % w/v of trichloroacetic acid. $n=3$, standard deviations <6.0 .

- a) old (5yr) unheated pigskin
- b) insoluble collagen
- c) old (5yr) unheated pigskin
- d) insoluble collagen
- e) gelatin
- f) meat, top round

Acid-solubility

The amount of soluble material in young and old unheated pigskin, without the action of added enzymes, is shown in Table 2 as the acid soluble and neutral salt soluble collagen (Johns, 1977). The hydroxyproline-solubility was 20.2% for young and 5.2% for old pigskin, values that are somewhat higher than those reported generally for skin by Johns (1977) and Ashgar & Henrickson (1982). The lower solubility of hydroxyproline in the old sample was expected since it is known that in collagen, while ageing *in vivo*, the acid labile intermolecular crosslink aldimine is converted into a stable form (Bailey *et al.*, 1974; Sims & Bailey, 1981). The pigskin material used also included non-collagenous proteins; for young pigskin 28.2% and for old 23.9% of the total protein. The difference of solubility in acid/buffer between total protein (nitrogen) and total collagen (hydroxyproline) (Table 2) is an estimate of solubilized non-collagenous proteins. For young pigskin the non-collagenous proteins amounted to 9.5% and for old pigskin to 5.1%. This showed that the non-collagenous proteins were not totally solubilized, although Johns (1977), indicated that these proteins could be extracted in acid.

The effect of pH on swelling and denaturation

Solubility after pepsin treatment at pH 1.5 showed quite high values; more than 84% for the unheated samples of young pigskin, tendon and insoluble collagen. These results are probably mainly due to the swelling effect of acid treatment. HCl has been shown to act with an osmotic swelling effect on collagen between pH 1-4, with a maximum at pH 2 (Gustavson, 1956). Apparently the interfibrillar space of collagen has to be sufficiently expanded for removing the steric hindrance of enzyme action (Bailey & Etherington, 1980). The lower values of pepsin solubility for the old unheated pigskin sample (Table 1) could be explained as a limited swelling capacity. A tighter structure is known to occur in mature collagen where the fibers are linked in register between penta-fibrils (Bailey, 1982). The swelling of the freeze-dried material used was obviously sufficient for the action of pepsin. Gustavson (1956) noted only 15% lower swelling values in HCl for dried hide when compared to a fresh sample.

Pepsin is known to solubilize collagen. However, it is generally accepted that the enzyme is not capable of disrupting the helical configuration of collagen but attacks only the telopeptides (Weiss, 1976). The way it acts on insoluble collagen is by cleaving one monomer close to its helix so that the intermolecular crosslink remains linked to the adjacent helix (Sims & Bailey, 1981). The lower solubilities obtained when using higher pH-values in the pepsin/HCl step (Fig. 2) could be due to an insufficient swelling effect or perhaps to a lowered pepsin activity. It has been indicated that denaturation of insoluble collagen can occur only after solubilization of the monomer (Etherington, 1977) and denaturation is necessary for further degradation by pepsin or pancreatin. The unheated samples solubilized at pH 1.5 by pepsin at 37°C should have denatured to a large extent since the denaturation temperature for the monomer is pH-dependent, decreasing from 37°C at pH 3.8 down to 32°C at pH 1-2 for, for example, soluble calf skin (Dick & Nordwig, 1966). It is generally accepted that the acidic pH of the stomach causes protein denaturation (Cheftel, 1979). For insoluble collagen HCl seems to be necessary for three consecutive steps 1) to induce swelling 2) to give an optimal pH for pepsin action and 3) to lower the denaturation temperature of the helix.

Pepsin and pancreatin action in degrading the monomer

The low TCA-solubility of nitrogen obtained after pepsin treatment of pigskin, tendon, insoluble collagen and gelatin samples (Table 1) indicated that only small amounts of the collagen monomer and gelatin had been degraded to peptides. These results could be explained by the fact that pepsin is known to cleave only at sites close to tyrosin and valine in gelatin (Weiss, 1976). These amino acids occur in very small amounts in the pigskin and tendon samples (Laser Reuterswärd, Asp & Björck, 1984) as well as in gelatin (Eastoe, 1967). The higher TCA-solubility values obtained for meat samples after pepsin digestion should be due to the larger amounts of these amino acids in meat proteins (Lawrie, 1979).

Low solubilities were obtained for both young and old pigskin samples after pancreatin treatment only (Table 2). After pre-swelling in HCl the solubilities increased significantly. The results of old unheated pigskin and insoluble collagen (Fig. 2) indicated that the same pH-value was critical for the solubilization by pepsin as well as by pepsin/pancreatin. The data also show that the collagen solubilized in the pepsin/HCl-step was further degraded by pancreatin to TCA-soluble peptides.

These results confirm the necessity of swelling for the susceptibility to enzyme action. Trypsin and chymotrypsin do not disrupt the collagen helix itself but attack only the non-helical telopeptides (Weiss, 1976). Table 2 also shows that the solubilities with and without TCA after HCl/pancreatin treatment were not significantly different. This indicates that the initial pancreatin solubilization was followed by denaturation of the monomer at 37°C and then by further degradation.

Figure 2 shows the TCA-solubilities at varying pH for insoluble collagen and gelatin. Gelatin, which is randomized and water-soluble, was almost completely degraded to TCA soluble peptides after pepsin/pancreatin treatment independent of the pH in the HCl/pepsin step. This was not true for insoluble collagen.

In conclusion, the results showed that pancreatin degrades all the pepsin solubilized collagen to TCA soluble peptides.

Heated samples

The data in Table 1 show that unheated pigskin and tendon samples were highly solubilized and degraded by pepsin/pancreatin. A positive effect of heat treatment on solubilization was only obtained for some samples and the overall effect was not significant.

Heat treatment performed at 74°C should have caused denaturation since insoluble fibers are known to denature at their shrinkage temperature. This occurs at 27°C higher than the denaturation temperature of soluble collagen, 36-41°C, but depends on the species (Bailey, 1968; Privalov, 1982).

Scalding of pigs is a mild heat treatment occurring in water at 60°C for 10 min. Some of the soluble pigskin collagen in immature pigs could be sufficiently labile to be denatured by scalding (Johns & Courts, 1977). The particles of the young scalded pigskin were bigger than the unscalded sample and looked more like the heated pigskin samples (Fig 1).

Since the particle size of the scalded and the heated samples were bigger than the corresponding unheated ones the possible positive effect of heating on solubilization could have been counteracted. Thus for the old pigskin sample the unexpectedly lower pepsin solubility after heating (Table 1) could probably also be due to

the bigger particle size of the heated sample.

A significant age effect was detected when comparing the solubilities of young and old heated pigskins. In old skin, as well as in young and old tendon, heat stable crosslinks are known to occur (Weiss, 1976; Sims & Bailey, 1981). In the present study, however, incubation with pepsin and pancreatin was more effective in solubilizing old tendon than old pigskin ($P < 0.01$). No significant age difference was obtained when solubilizing tendons at pH 1.5 with pepsin (Table 1). This is not in accordance with the findings of Etherington (1977) who obtained solubilities of 80% for 2 year old and 65% for 10 year old insoluble collagen from Achilles tendon. The conditions were different since he used pepsin/acetic acid (2 hr, 37°C) at pH 2.65.

Other studies

Neumann & Tytell (1950) obtained high solubility values, 95%, after pepsin treatment (pH 2, 37°C, 24hr) of the Achilles tendon from cattle. Their results are in accordance with the value for insoluble collagen in the present study. Franke (1964) did not notice any differences in the pepsin digestibility of raw and heated pigskin. Both were about 97% digestible but no specification was given on scalding or incubation conditions. The low digestibility, 38% and 48%, obtained by Loewit (1967) on raw pigskin and tendon after treatment in pepsin at pH 2.7 at 37°C for 3hr, followed by pancreatin for 14hr, could probably depend on the high pH-value used. The large differences obtained by Harkness *et al.* (1978), in the solubility of rat tail and bovine tendon, 97% and 37%, after treatment with pepsin/HCl (pH 1.5, 37°C, 2hr) were explained as a possible age effect, but this does not agree with the present results. Harkness *et al.* also compared human gastric juice to (unspecified) pepsin on the solubility of rat tail tendon, but did not find any differences.

CONCLUSIONS

Unscalded pigskin and unheated tendon were highly solubilized (75-94%) when incubated with pepsin and hydrochloric acid at pH 1.5 and pancreatin at pH 6.8 followed by TCA precipitation. A significantly lower solubility was obtained for pigskin from an old animal than from a young one. No age effect was recorded for tendons from calves and cows.

The pH during incubation with pepsin and hydrochloric acid was critical for the solubilization of the collagenous samples but not for the gelatin and meat samples. At a pH of about 2.5 the solubility started to decrease. Further treatment with pancreatin only slightly increased the solubility but had a prominent effect on the TCA solubility.

Solubilization of insoluble collagenous samples such as unheated pigskin and raw tendon after pepsin and pancreatin treatment indicate that digestibility could be high *in vivo*. Confirmation of these data should be performed *in vivo* however, since degradation of insoluble collagen is very sensitive to variations of pH during incubation with pepsin in hydrochloric acid.

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Protein digestibility of pigskin and bovine tendon in rats

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ABSTRACT

The protein nutritional value of pigskins and bovine tendons, with collagen contents of 75 and 95% respectively, was evaluated using amino acid analyses and nitrogen balance experiments on rats. The amino acid compositions showed that pigskins contain somewhat higher amounts of tryptophan, methionine, cystine and tyrosine than tendons. The amounts of several essential amino acids were low. The true digestibilities were for young (4 month), unscaled pigskin 96.1%, old (5 year), unscaled pigskin 92.5%, calf tendon 97.3% and cow tendon 92.4%. Small but statistically significant differences due to the animal age were observed. Insoluble collagen, extracted from bovine tendon, had a digestibility of 95.2%. Wet heat treatment of old pigskin and cow tendon samples at 74°C for 30 min significantly increased the digestibilities to 96.3 and 97.3%, respectively. The results indicate that prior denaturation of collagen, as obtained by, for example, heat treatment, is not necessary for the digestion of pigskin and tendon in rats.

INTRODUCTION

From a nutritional point of view muscle proteins are considered to be more rapidly and easily digested than collagen (Brüggeman *et al.* 1964; Davidson *et al.* 1979) and many plant proteins (Snook, 1973). Native collagen is considered more or less indigestible (Loewit, 1970; Cheftel, 1977; 1979; Kies, 1981; Ashgar & Henrickson, 1982) but heating and the conversion to gelatin makes it susceptible to enzymes and thus digestible (Paul, 1972; Rogowski, 1980; Bender, 1980; Prandl, 1980; Ashgar & Henrikson, 1982). Gelatin has a digestibility of 90% *in vivo* (Mauron, 1973). Only a few *in vivo* investigations on the digestibility of collagen have been performed, evaluated as apparent digestibility in rat assay. The collagenous materials used in these studies, however, cannot be considered native since they were either limed or partly heated. Thus Whitmore *et al.* (1975) found limed cattlehide to be 90% digestible. Happich *et al.* (1975) showed a digestibility of 88% for partially defatted chopped beef containing 50% collagen and heated to about 50°C. Raw, probably scalded, pigs ears with high amounts of collagen were 88% digestible (Vaughn *et al.* 1979).

In a previous study (Laser Reuterswärd *et al.* 1982) it was shown that the true digestibility of a mixture of raw pigskin and beef containing 50% collagen was 99%. The pig used was six months old and the carcass was scalded according to normal practice. Scalding may denature some of the soluble collagen in biologically immature pigs (Johns & Courts, 1977). It is known that insoluble collagen from a mature animal shows an increasing resistance to solubilizing agents,

including pepsin, due to the stabilization of intermolecular crosslinks (Weiss, 1976; Sims & Bailey, 1981). No studies of the *in vivo* digestibility of collagen from mature animals are available in the literature. However, Harkness *et al.* (1978) found that the digestibility of rat tail tendon, evaluated in a rat assay, was drastically reduced after the introduction of artificial crosslinks.

The aim of the present study was to evaluate the true digestibility of highly collagenous materials such as pigskin and bovine tendon in a rat assay. The effects of animal age as well as the scalding of carcasses and the cooking of pigskin and tendon samples were of special interest.

MATERIALS AND METHODS

Sample preparation

Pigskins

Skins were removed immediately after slaughter from a 4 month old pig and a 5 year old sow. The pigskins were manually shaved with a razor. Another pigskin was taken from a 6 month old pig which had been scalded according to slaughterhouse procedures in water at 60°C for 10 minutes. All the pigskins were freed of meaty parts and visible fat. The samples were finely cut in a Moulinex blender. Half of the samples were vacuum packed in plastic bags in thicknesses of less than 1 cm, heated in a water bath at 74°C $\pm$ 1°C for 30 min and immediately chilled. The heated samples were finely recut in a Moulinex blender. The unheated and heated samples were lyophilized. The unheated samples were minced in a knife homogenizer (Janke & Kunkel, GFR) and the heated samples in a Moulinex blender. The unscalded young and old pigskins, when surveyed under a low magnification microscope, appeared similar and were porous and crystalline-like having a tight structure. Except for the unscalded young and old pigskins, the other pigskin samples were mixed with a portion of maize starch and ground in a hammer-mill with a screen of 0.8 mm before being mixed into the diets.

Tendons

Achilles tendons were collected from forty 7 month old calves and from forty cows with an average age of 6.6 years (range 4 to 12.5). The calf tendons were collected 24 hours and the cow tendons 1-6 days after slaughter. The tendons were freed of meaty parts and visible fat and frozen for not more than 2 months. After thawing, the tendons were cut into smaller pieces. The wet calf and cow samples were ground to pass a screen of 3 mm and the samples were immediately chilled. The temperature of the calf sample reached 32°C and the cow sample 44°C during grinding. Half of the cow sample was packed and heated as described for the pigskin samples above and cut in a Moulinex blender. The unheated and heated samples were lyophilized and homogenized in a hammer-mill. When surveyed under a low magnification microscope the unheated hammer-milled calf and cow samples appeared similar with a porous and crystalline-like structure. The heated hammer-milled cow sample showed tighter particles.

Insoluble collagen from bovine Achilles tendon, prepared according to Einbinder & Schubert (1951), was purchased from Sigma Chem. Comp., USA.

The tendon samples were mixed into the diets without any further preparations.

Chemical analyses

Nitrogen was analyzed according to Kjeldahl and hydroxyproline analyzed on a Technicon Auto-Analyzer according to Stegemann (1958) as modified by Weber (1973). Calculations of collagen and protein contents were made according to Laser Reuterswärd *et al.* (1982).

Amino acid analyses were performed on duplicate samples after acid hydrolysis in 6N HCl at 110°C for 24h. Cystine and methionine were determined after performic acid oxidation. Separation was performed on a Durrum D-500 Analyzer. Tryptophan was hydrolyzed with 3M mercaptoethanesulfonic acid at 110°C for 24h and analyzed on a Beckman 120B Amino Acid Analyzer.

Nitrogen balance experiments

Nitrogen balance studies were performed on growing rats, male Sprague-Dawley, Anticimex, Sweden. Each diet was tested on five rats. The animals were kept individually in metabolic cages according to Eggum (1973). After four days of acclimatization the urine and faeces were collected during a five-day balance period. Temperature was maintained at 25°C and relative humidity at 55%. The diet was composed according to Eggum (1973) with modified contents of vitamins and minerals according to Forsum *et al.* (1973). The composition of the diet was as follows (% of dry matter): protein corresponding to 1.5% nitrogen, maize oil 5%, cellulose powder 5%, mineral mixture 4.8%, vitamin mixture 0.8%, cholin chloride 0.2%, saccharose 10% and maize starch up to 100%.

The diets were supplemented with L-Methionine (Biochemische Zwecke, Merck), L-Tryptophan (chromatographically homogenous), L-Isoleucine and L-Histidine×HCl (all from BDH Chem. Ltd., England). The levels of these four amino acids in the diets were for pigskin 0.20%, 0.06%, 0.04% and 0.06% and for tendons 0.15%, 0.10%, 0.10% and 0.10%, respectively. A diet containing ANRC Reference Protein High Nitrogen Casein (Sheffield Chem., USA) supplemented with 0.22% methionine (dry matter) was used as a reference.

The nitrogen contents of the feed, urine and faeces were analyzed by the Kjeldahl method. Calculations of true digestibility, biological value and net protein utilization were performed with the Thomas-Mitchell equations as described by Eggum (1973). Values for endogenous and metabolic nitrogen were obtained from experiments with a diet containing fat extracted, lyophilized, hens egg corresponding to 0.72% nitrogen (dry basis). Mean values obtained during the five-days balance period were for faeces, 60.5 mg and urine 76.2 mg.

Statistical evaluation

Means and standard deviations were calculated and analyzed by Student's t-test (Bailey, 1981).

RESULTS AND DISCUSSION

Amino acid composition

The amino acid composition as well as the collagen and protein content are shown in Table 1. The protein in the tendon samples contained more collagen than the protein in pigskins, the value for pigskin agrees with the results reported by Johns (1977). The variations in total protein content were mainly due to the varying amounts of fat.

The amino acid composition of the different pigskins was similar. No data on the complete amino acid composition of intact pigskin is available in the literature. However, when compared to results obtained by Eastoe (1967) on pure gelatin the contents of glycine, proline and hydroxyproline were lower in pigskin. In contrast considerably higher amounts of cystine, methionine, and tyrosine were found in the intact pigskin. The tryptophan content was about 0.15 g/100 g protein in pigskins but this amino acid is lacking in gelatin.

The amino acid composition of the four tendon samples (Table 1) was similar and in good agreement with the pattern reported for cattle Achilles tendon by Eastoe (1967).

In the tendons, the content of lysine was considerably lower and that of hydroxylysine higher than the corresponding values in pigskins. However, the sum of lysine and hydroxylysine was fairly constant, in pigskins 4.8 to 5.3 and in tendons 4.8 to 5.5g/18gN, very much in line with the findings of Eastoe (1967).

An approximate 28% loss of methionine was found for the scalded/heated pigskin when compared to the scalded one. Loss of methionine during heat treatment of pork has been reported previously by Donóso *et al.* (1962).

The chemical scores for several amino acids in pigskins and tendons were very low when calculated in relation to the need of the rat according to the Food and Nutrition Board (1974) as cited by Steinke (1979). For pigskins and tendons the chemical scores were for tryptophan 11 and zero, respectively and for isoleucine, methionine/cystine and histidine the values were all below 31 and 33 respectively. Supplementation with these amino acids was performed to provide a better amino acid balance and thus ensure a more reliable determination of digestibility. The amounts of amino acids supplemented could be considered negligible compared to the total protein content and would not influence the evaluation of digestibility.

Nitrogen balance experiments

Table 2 shows results of true protein digestibility. All pigskins and tendon samples were digested to at least 92%.

A significant difference in digestibility was found between young and old pigskins as well as between calf and cow tendon ($P<0.01$).

Heat treatment of the old pigskin and the cow tendon samples resulted in a significant increase in digestibility, ($P<0.05$; $P<0.01$, respectively). However, for the two young pigskin samples no significant effect of heat treatment was found. Scalding had no significant effect on the digestibility when comparing the two young pigskin samples. The digestibilities of the heated old pigskin and cow tendon samples were not significantly different from the corresponding unheated young pigskin and calf tendon.

Table 1. Amino acid compositions, protein and collagen content of pigskin and tendon samples from animals of different ages. Amino acids expressed in g/18 gN, corresponding to 100 g protein. Heat treatment was performed on wet material at 74 °C for 30 min.

	PIGSKIN					TENDON				
	4 m unscalded	4 m heated	6 m scalded	6 m scalded/ heated	5 y unscalded	5 y heated	Calf raw	Cow raw	Cow heated	Insoluble collagen
Aspartic acid	6.8	6.9	7.3	6.6	6.9	7.2	6.1	5.9	6.3	6.3
Threonine	2.4	2.4	2.4	2.3	2.3	2.4	2.1	1.9	2.1	2.0
Serine	4.2	4.3	4.4	4.1	4.4	4.6	3.5	3.4	3.6	3.6
Glutamic acid	11.2	11.4	12.0	10.8	11.4	12.0	10.2	9.8	10.5	10.6
Proline	12.5	12.9	14.4	13.7	13.0	14.7	12.5	12.4	13.0	13.9
Glycine	21.4	21.6	24.0	22.1	21.2	22.9	21.7	21.7	22.9	25.4
Alanine	8.8	9.2	10.1	9.1	9.2	10.0	9.3	9.2	9.7	10.4
Cystine	0.57	0.76	0.38	0.25	0.48	0.52	tr	0.18	0.20	tr
Valine	3.1	3.1	3.2	2.9	3.0	3.1	2.6	2.5	2.7	2.6
Methionine	1.5	1.7	1.6	1.2	1.3	1.1	0.82	0.78	0.89	0.97
Isoleucine	1.9	1.8	1.8	1.6	1.8	1.8	1.7	1.6	1.7	1.6
Leucine	4.1	4.1	4.1	3.7	3.9	4.0	3.6	3.4	3.6	3.3
Tyrosine	1.3	1.3	1.3	1.1	1.2	1.2	0.81	0.78	0.72	0.69
Phenylalanine	2.4	2.5	2.6	2.4	2.4	2.5	2.1	2.0	2.1	2.1
Histidine	0.93	1.0	1.1	0.98	0.95	0.98	0.77	0.70	0.75	0.76
Lysine	3.9	4.2	4.4	4.1	4.1	4.3	3.2	3.1	3.4	2.9
Hydroxylysine	0.86	0.78	0.89	0.93	0.71	0.72	2.2	1.7	1.8	2.6
Arginine	8.2	8.7	9.3	8.5	8.7	9.3	8.5	8.2	8.9	9.0
Ammonia	0.97	0.90	0.95	0.84	0.93	0.91	0.77	0.81	0.78	0.80
Hydroxyproline	10.1	10.4	10.4	10.7	11.0	11.2	13.2	13.7	13.5	13.6
Tryptophan	0.14	0.09	0.15	0.16	0.18	0.17	0.07	tr	tr	0.00
TOTAL	107.2	110.0	116.6	108.0	109.1	115.3	105.6	103.8	109.2	113.1
Protein/dry weight %	47.4	52.1	58.1	63.5	70.2	70.8	93.5	99.7	97.2	102.2
Collagen as % of protein	71.8	71.7	71.3	73.7	76.1	77.6	92.5	96.7	94.8	96.4

Table 2. Mean values of true digestibility for pigskin and tendon samples from animals of different ages. $n = 5$ and standard deviations within brackets. Wet heat treatment of samples no 4, 6 and 9 was performed at 74°C for 30 min.

	True Digestibility %	Significance ²⁾							
		1	2	3	4	5	6		
<u>Pigskin</u>									
1. Young (4 m) unscaled	96.1 (1.2) ¹⁾	—	ns	ns	ns	**	ns		
2. Young (4 m) heated	97.4 (1.5)		—	ns	ns	**	ns		
3. Young (6 m) scaled	97.5 (1.9)			—	ns	**	ns		
4. Young (6 m) scaled/heated	97.7 (1.3)				—	***	ns		
5. Old (5 y) unscaled	92.5 (1.7)					—	*		
6. Old (5 y) heated	96.3 (2.5)						—		
<u>Bovine Achilles Tendon</u>									
7. Calf (7 m) raw	97.3 (1.0)							7	8
8. Cow (6.6 y) raw	92.4 (2.2)							—	**
9. Cow (6.6 y) heated	97.8 (2.0)								—
10. Insoluble collagen	95.2 (2.2)								ns
Reference Casein (pigskin)	99.9 (1.8)								ns
Reference Casein (tendon)	99.1 (0.8)								—

¹⁾ $n = 4$

²⁾ ns = not significant

* = $P < 0.05$

** = $P < 0.01$

*** = $P < 0.001$

The feed residues, in % of the diet offered to the rats, were for the pigskin samples between 21-45%, the highest value obtained with the young, scaled pigskin. With the tendon samples, feed residues were 9-14% and with casein 1%. According to Eggum (1973), animals with feed residues tended to give lower digestibility values than animals without feed residues, but the difference was not statistically significant. Although the feed residues in the present study were in some cases quite large, this would not have caused overestimation of the digestibility.

Some of the samples used in the present study were previously studied in an *in vitro* system evaluated as nitrogen solubility. Incubation was performed at 37°C with pepsin at pH 1.5 and pancreatin at pH 6.8 followed by precipitation with trichloroacetic acid (TCA). With the young and old unscaled pigskins the TCA-solubility values were 94% and 75% respectively. With the four tendon samples the solubilities were all about 92% (Laser Reuterswärd, 1984).

The values obtained *in vitro* are thus in the same range as the *in vivo* values except for the old pigskin sample where a much lower *in vitro* value was obtained. A high degree of solubilization *in vitro* could therefore be considered as an indication of high digestibility *in vivo* for these kinds of samples.

It is known that as animals age, collagen fibers become more stable assessed by tensile strength, isometric tension, swelling and solubility in hot water and acid.

This is due to the stabilization of the intermolecular crosslinks (Weiss, 1976; Sims & Bailey, 1981; Bailey, 1982). Increased resistance to digestion by, for example, pepsin in mature animals has also been reported (Weiss, 1976) resulting in decreased solubility in pepsin/acetic acid for insoluble collagen from old animals (Etherington, 1977). The present *in vivo* study indicates that despite an increase in age, high digestibilities were obtained under conditions occurring in the gastrointestinal tract of the rat. In contrast, when artificial crosslinks were introduced, the *in vivo* digestibility of rat tail tendon decreased from 102% to 39% (Harkness *et al.* 1978).

The present results also show that the sample of extracted insoluble collagen from tendon had a digestibility of 95.2%, a value between those of calf and cow tendons but not significantly different from either of them. Mucopolysaccharides and soluble proteins from the collagen tissue matrix were extracted in this sample. Etherington (1977) pointed out that proteoglycans and mucopolysaccharides may have a stabilizing role complementary to the intermolecular crosslinks of collagen and that these carbohydrates may restrict access of an enzyme to the non-helical telopeptide region. However, the present findings indicate that the presence of the tissue matrix of pigskin and tendon did not limit digestion *in vivo*.

In skin and tendons the collagen fibers are mainly (about 90%) insoluble (Johns, 1977; Carmichael & Lawrie, 1967) and insoluble collagen denatures at a temperature of about 65°C when wet heated (Bailey, 1968). The temperatures obtained during grinding of the wet calf and cow samples were thus well below the denaturation temperature.

The present results show that the unheated samples as well as the heated ones were highly digestible. This indicates that prior heat treatment is not necessary since solubilization and gelatinization of the insoluble collagen obviously occur during digestion *in vivo*. This is discussed in more detail elsewhere (Laser Reuterswärd, 1984; Laser Reuterswärd & Fabiansson, 1984).

The biological values after supplementation for all pigskin and tendon diets were between 44.3 and 52.2%. No significant differences were found between pigskin samples or between tendon samples. Since the true digestibility values were quite high the net protein utilization values were found to be close to that of the biological values, between 41.4 to 50.6%.

It could be concluded that protein in pigskin and bovine tendon was digested to at least 92% *in vivo* irrespective of animal age and prior heat treatment. Significant differences due to age were obtained; young pigskin and calf tendon were somewhat more digestible than old pigskin and cow tendon. Prior heat treatment of tissues from old animals increased the digestibilities to the same level as seen in young animals.

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In vivo digestibility of insoluble collagen from bovine tendon as influenced by the inhibition of gastric acid secretion

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ABSTRACT

Insoluble collagen (bovine Achilles tendon), gelatin and meat were each mixed with whole egg protein (about 50% of the nitrogen) and fed to rats. True digestibility was evaluated after inhibiting gastric acid secretion with omeprazole. Insoluble collagen, supplemented with small amounts of essential amino acids, was also given but without inhibiting gastric acid secretion. Egg protein was fully digested and not affected by inhibition. The digestibility of insoluble collagen/egg was 85%, corresponding to 71% digestibility for insoluble collagen alone. This was significantly lower than the value of 95% obtained without inhibiting the gastric acid secretion. The digestibilities for gelatin/egg were 99% and meat/egg 98%. The inhibition of gastric acid secretion thus did not affect the digestion of gelatin and meat.

INTRODUCTION

Collagen normally constitutes a very small part of the total intake of food proteins. The content of intramuscular collagen varies from about 2 to 20% of the protein in meat (Lawrie, 1979). In some products, as for example Swedish sausages, 35% of the protein can be of collagenous origin (Fuchs & Kuivinen, 1980). Native collagen is considered to be more or less indigestible (Loewit, 1970; Cheftel, 1977; 1979; Kies, 1981; Ashgar & Henrickson, 1982) but denaturation and solubilization to gelatin, as for example after cooking, makes it susceptible to proteolytic enzymes and thus digestible (Paul, 1972; Rogowski, 1980; Bender, 1980; Prandl, 1980; Ashgar & Henrickson, 1982). It has, however, been reported that also unheated samples such as collagenous unscaled pigskin and raw bovine tendon derived from both young and old animals have a true digestibility of more than 92% in rats (Laser Reuterswärd *et al.* 1984).

The initial step in the normal digestion of food proteins is their denaturation at acidic pH and the action of pepsins in the stomach (Gitler, 1964; Bender, 1978; Silk & Dawson, 1979). *In vitro* studies have shown that pepsin can, to a large extent, solubilize collagen fibers by cleaving peptide bonds in the non-helical telopeptide region (Weiss, 1976). In a previous *in vitro* study solubilization of old unscaled pigskin (75% collagen of the protein) and insoluble collagen (extracted from bovine tendon) were both found to be highly pH-dependent during incubation with pepsin in hydrochloric acid. When the pH was increased a prominent decrease in solubility was obtained already at pH 2.5 and further treatment with pancreatin had only a small effect on solubilization (Laser Reuterswärd, 1984a). Denaturation of insoluble collagen fibers occurs only after solubilization (Eth-

erington, 1977). The denaturation temperature is around 39°C (Bailey, 1983) but is pH-dependent and decreases at a lower pH (Dick & Nordwig, 1966). Experiments *in vitro* thus indicate on a strong relationship between pH and collagen solubilization and denaturation.

The present study was performed in order to establish whether gastric acid secretion was critical for the digestion of insoluble collagen *in vivo*. The gastric acid secretion was inhibited by a drug, omeprazole.

MATERIAL AND METHODS

Materials

Beef, *M. psoas major*, was freed of visible fat and connective tissue, minced in a Moulinex blender and freeze-dried. Gelatin from swine skin (175 Bloom) and insoluble collagen from bovine Achilles tendon, prepared according to Einbinder & Schubert (1951), were purchased from Sigma Chem. Comp., USA. ANRC Reference Protein High Nitrogen Casein (Sheffield Chem., USA) was used as the reference protein. Whole egg was fat extracted, lyophilized and used for determination of metabolic nitrogen.

Rat assay

True digestibility was evaluated by nitrogen balance studies on growing rats. Male Sprague-Dawley rats (initial weight of 85g) from Anticimex, Sweden, were placed alone in metabolic cages according to Eggum (1973). A four days adaption period was allowed. The temperature was held at 26°C and the relative humidity at 65%. Every 12th hour light and darkness were exchanged. Faeces were collected in 5% H₂SO₄ during the five-day balance period with experimental diets.

Omeprazole (Hässle, Sweden), inhibiting gastric acid secretion, was administered by intragastric intubation twice a day at 8 a.m. and 8 p.m., 0.5 ml of a suspension of 8 µmol/ml. The intubation was performed after light diethyl ether anaesthesia. Intubation was performed from the second day of the adaption period and throughout the experiment.

A diet based on whole egg protein at a level of 0.72% nitrogen (dry basis) was administered to determine metabolic nitrogen under the omeprazole conditions. Seven rats were used in this group. Studies were further performed with diets containing 1.5% nitrogen (dry basis), 0.72% of which was from whole egg protein and the remainder from gelatin, insoluble collagen or meat. Six rats were used in each of these groups. For comparison, reference casein supplemented with methionine (0.22% of the diet) and insoluble collagen supplemented (dry matter) with 0.15% methionine and 0.10% each of tryptophan, isoleucine and histidine were tested, both at a level of 1.5% nitrogen (dry matter). Five rats were used in these groups. Reference casein and insoluble collagen were tested without using omeprazole and correction was then made for metabolic nitrogen determined under corresponding conditions. L-methionine (Biochemische Zwecke), L-Tryptophan (chromatographically homogenous), L-Isoleucine and Histidine×HCl(all from BDH Chem. Ltd., England) were used for supplementation. The diets were composed according to Eggum (1973) with modified contents of minerals and vitamins according to Forsum *et al.* (1973). Maize starch was used as the carbohydrate source and to adjust dry matter content.

Chemical analyses

Nitrogen contents of original samples, diets and faeces were analyzed using the Kjeldahl method. Calculation of true digestibility was performed with the Thomas-Mitchell equation as described by Eggum (1973).

Hydroxyproline was analyzed on a Technicon Auto-Analyzer according to Stegemann (1958) as modified by Weber (1973). Calculations of collagen content were made according to Laser Reuterswärd *et al.* (1982).

Stomach content

Each rat was killed in a cage, on the 5th day, by diethyl ether, 12 hours after receiving the last omeprazole dose. The stomach was removed and put into 20 ml of de-aired ice cold water, cut open and emptied of its contents by mincing carefully. The pH was measured and the H^+ concentration was estimated by titration using de-aired 0.001N NaOH to a final pH of 8.00 (Davenport, 1977) in duplicates.

RESULTS AND DISCUSSION

Effects of omeprazole

Studies of Larsson *et al.* (1983) have indicated that omeprazole inhibits gastric acid secretion by blocking the parietal cell proton pump - the H^+ , K^+ - ATPase. Omeprazole has a very long duration of inhibition, a feature unique among gastric antisecreting agents. Repeated oral administration of omeprazole, at the conditions in the present study (3 days of adaption, 40 μ mol/kg, every 12th hour) results in anacidity for 6 to 8 hours after each dose and at least 80% inhibition of gastric acid output over 24 hours (Carlson, personal communication).

The amount of metabolic nitrogen obtained for egg protein when using omeprazole was 71.0 (8.4) mg which was not significantly different from the value of 63.9 (2.8) mg obtained under ordinary conditions. (Standard deviations within brackets).

Table 1 shows pH and amount of titrable acid in the stomach contents suspended in 20 ml water. The omeprazole group ($n=24$) had a significantly higher ($P<0.01$) pH value than the reference group ($n=5$) and the amount of acid was significantly ($P<0.001$) lower. These values were estimated 12h after the last omeprazole dose i.e. when the inhibitory effect of the drug could be expected to be minimal.

True Digestibility

The meat sample contained 2.4% collagen of the total protein. The amino acid compositions of insoluble collagen and gelatin were similar (Laser Reuterswärd, 1984b).

Table 1 shows the results of true digestibility. The calculated value for meat, assuming egg protein 100% digestible, was 97.1%. The high values for egg and meat proteins agree well with previously reported findings by Mauron (1973) using rat assay under normal conditions evaluated with a nitrogen balance method. Since egg and meat are main protein sources in normal diets the present results agrees with the findings that total gastrectomy does not increase nitrogen in the

Table 1. Influence of omeprazole inhibition on gastric acid production and true digestibility of rats fed different proteins. Standard deviations within brackets. pH and H^+ concentration were measured after dilution of stomach content in 20 ml water, at the time of the completion of the rat experiment.

Diet protein	Nitrogen in diet %	n	Gastric acid secretion		True Digestibility %
			pH	H + ¹⁾ ml	
<u>Without omeprazole</u>					
Casein (reference)	1.50	5	3.5 (0.1)	39.0 (18.5)	100.0 (0.3)
Insoluble collagen	1.50	5	—	—	95.2 (2.2)
<u>With omeprazole</u>					
Whole egg	0.72	7	6.0 (1.2)	16.4 (2.3)	99.9 (2.4)
Meat/Whole egg	0.78/0.72	6	4.5 (1.2)	13.7 (3.8)	98.4 (2.0)
Gelatin/Whole egg	0.78/0.72	5	5.8 (1.2)	18.8 (15.6)	99.4 (1.9)
Insoluble collagen/ Whole egg	0.78/0.72	6	5.4 (1.0)	11.9 (7.6)	85.0 (3.2)

¹⁾ Titrated against 0.001 NaOH to pH 8.0

faeces of rats (Welbourn & Doggart, 1956) and that partial gastrectomy in man does not increase faecal nitrogen (Taylor, 1968; Silk & Dowson, 1979; Freeman *et al.* 1979; Johnson, 1981). The digestibility value calculated for gelatin was 98.8%. Mauron (1973) reported 90% digestibility under normal conditions.

For the insoluble collagen sample a significant ($P < 0.001$) decrease in digestibility due to the effect of omeprazole was observed (Table 1). The calculated true digestibility of insoluble collagen was 71%. In an *in vitro* study it has previously been shown that solubilization of insoluble collagen (same material as in this study) was highly pH-dependent during incubation with pepsin in hydrochloric acid (Laser Reuterswärd, 1984a). Further treatment with pancreatin had only a small effect on solubilization. The solubility at pH 1.5 was 90%, but started to decrease at pH 2.5. For the gelatin and meat samples a much less pronounced effect of pH was obtained *in vitro*. At pH 1.5 both proteins were completely solubilized and at pH 4 the gelatin sample was 95% soluble and the meat sample 80% soluble. Data from the present study and the *in vitro* study (Laser Reuterswärd, 1984a), also indicate that pH has a greater influence on the digestion of insoluble collagen than on that of meat proteins and gelatin.

Effects of hydrochloric acid

The pH in stomach during digestion in humans varies depending on individuals and their diet composition (Gitler, 1964; Davenport, 1977). In general the action of hydrochloric acid on proteins is related to denaturation (Gitler, 1964; Bender, 1978; Silk & Dawson, 1979) and peptide bonds shielded inside the native protein become more accessible to proteases after denaturation (Gitler, 1964; Cheftel, 1979). However, four different effects of hydrochloric acid could be of importance for digesting collagen in the gastrointestinal tract of rats: 1) breaking acid labile crosslinks, 2) swelling of fibers, 3) providing an optimum pH for pepsin activity and 4) denaturation at a lowered denaturation temperature.

Acid labile crosslinks

Hydrochloric acid could break one of the intermolecular crosslinks of collagen, the 'aldimine', which is labile against acid. The 'aldimine' occurs in tendon in equal proportions to the other major crosslink, the 'keto', but the latter form is stable against acid (Weiss, 1976; Sims & Bailey, 1981). However, the importance of this cleavage is probably relatively small since less than 10% of the collagen is acid soluble in tendon (Carmichael & Lawrie, 1967).

Swelling

Hydrochloric acid has a swelling effect on hide collagen and this has been shown to be maximal at pH 2, *in vitro* (Gustavson, 1956). Above pH 4.5 insoluble collagen is totally resistant to degradation by cathepsins *in vitro*, probably due to the lack of swelling, which was indicated to be a prerequisite for the action of an enzyme within the interfibrillar space (Bailey & Etherington, 1980). The action of pancreatin and elastase on insoluble collagen, which is known only to occur in the non-helical telopeptide region (Weiss, 1976; Bailey & Etherington, 1980), would therefore be weak since a neutral pH is found in the small intestine. Collagenases, acting at neutral pH, are the only enzymes cleaving the collagen molecule in the intact helix (Bailey & Etherington, 1980). Fullmer *et al.* (1966), however, stated that they could find no collagenolytic activity in the alimentary tract of rats. The inhibition of hydrochloric acid secretion in the present study would have resulted in a reduced swelling effect of the collagen fibers. The digestibility value of 71% found, however, indicated that the swelling effect was nevertheless relatively sufficient for pepsin action.

Optimum pH for pepsin activity

A third effect of hydrochloric acid could be to provide an optimum pH for pepsin activity for degradation of the collagen fibers. Pepsin action on undenatured collagen is known only to occur in the non-helical region (Weiss, 1976). The inhibition of hydrochloric acid by omeprazole and the increase of pH should have resulted in a change of the conditions for pepsin activity. Pepsinogen is converted to pepsin in solutions which are more acidic than pH 5 and the optimal pH of pepsin is around pH 2 for most proteins (Taylor, 1968). However, several mammalian gastric proteinases with different pH-optima have been found (Foltman, 1981). Degradation of fibrous bovine tendon collagen by human pepsin 1 and 3 active up to pH 3.5 and 3.8, respectively, at 37°C has been reported. Pepsin 1 is a minor and pepsin 3 a major pepsin in humans (Etherington *et al.* 1980). Parapepsin I, a minor component of gastric proteinases (Foltman, 1981) has shown high activity towards gelatin (Ryle & Porter, 1959) and is probably equivalent to gelatinase according to Etherington & Taylor (1967). However, this enzyme does not have a high activity against collagen (Steven, as cited by Ryle, 1964). It is therefore possible that in the gastrointestinal tract of rats gastric proteinases which are also active at a higher pH than 2 are important in solubilizing collagen.

Denaturation

Denaturation of collagen fibers can only occur after solubilization of the monomers (Etherington, 1977). The fourth effect of hydrochloric acid on collagen degradation could therefore be that of denaturation/gelatinization of pepsin solubilized mono-

mers (tropocollagen). The denaturation temperature of tropocollagen, i.e. where half of the helix unfolds into random chains, is around 39°C for mammalian collagen according to Bailey (1983). The temperature for different soluble collagens, i.e. tropocollagen, varies between 36 and 41°C at pH 3.7 depending on the species (Bailey, 1968; Privalov, 1982). However, the exact denaturation temperature for bovine tendon used in the present study is not available in the literature. Moreover, the denaturation temperature is pH dependent, decreasing from 36°C at pH 3.7 to 32°C at pH 2 for soluble calf skin (Dick & Nordwig, 1966). The body temperature of rats is 37 to 38°C (Falkmer & Waller, 1972). Inhibition of hydrochloric acid, as in the present study, could thus have resulted in a less extensive denaturation after solubilization compared to the control sample. However, in the *in vitro* study of the same material it was shown that all insoluble collagen solubilized by pepsin was also degraded into trichloroacetic acid soluble peptides by further treatment with pancreatin. The degradation of collagen was thus independent of the pH of hydrochloric acid (Laser Reuterswärd, 1984a). It is therefore probable that the denaturation of solubilized collagen is not a limiting step in the digestion of collagen fibers. This indicates that swelling and the solubilization by gastric proteinases probably are the most critical factors for the digestion of insoluble collagen.

CONCLUSIONS

In conclusion, the secretion of hydrochloric acid *in vivo* was shown to be of importance for the digestion of insoluble collagen but not for gelatin and meat proteins. The implication of the present data for other collagens needs further study, as well as the importance of pH for the digestion of insoluble collagen in man.

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Urinary excretion of hydroxyproline in rats fed hydroxyproline-rich diets

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ABSTRACT

Urinary excretion of hydroxyproline was studied during nitrogen balance experiments with rats fed casein, meat, and hydroxyproline-rich diets such as gelatin, insoluble collagen and pigskin as the main protein source. Hydroxyproline in the urine was analyzed with and without prior hydrolysis to determine total and free amounts, the difference indicating amounts of peptide bound hydroxyproline. The levels of hydroxyproline from rats fed a casein diet were considered as being derived from basal degradation of tissue collagen and were used for correction of the levels obtained from the test diets. The excretion of total and free hydroxyproline in rats fed the meat diet did not significantly differ from the corresponding values of the casein control. Elevated excretions were found after ingestion of the hydroxyproline-rich diets. The difference between total and free amounts was similar with all diets, indicating that the excretion of hydroxyproline containing peptides did not change. At most 2.5% of the ingested hydroxyproline was excreted as free imino acid in the urine.

INTRODUCTION

Native collagen is considered to be more or less indigestible (1, 2) and it has been put forward that in order to render the molecule digestible denaturation and solubilization to gelatin, as for example by cooking, is necessary (3, 4, 5). Thus, gelatin was found to be 90% digestible of rats (6). However, a recent study of rats indicated that more than 92% of the protein in native collagenous materials such as tendon and pigskin was digestible without prior heat treatment (Laser Reuterswärd *et al.* 1984, submitted for publication).

Denatured collagen has an extremely unusual primary sequence with high levels of proline, hydroxyproline and glycine, and is therefore a poor substrate for the majority of the exopeptidases and many nonspecific endopeptidases. This results in large peptide fragments not easily susceptible to further attack (7). The intestinal epithelium, where, for example, proline dipeptidases are found, appears to be the principal site for terminal digestion of luminal peptides (8). However, evidence exists, that after ingesting gelatin, peptides containing hydroxyproline are absorbed intact and excreted in the urine of humans as well as of rats (9, 10, 11). In a study by Prockop *et al.* (12), up to 7% of the hydroxyproline in gelatin was excreted by humans as peptides in the urine.

No reports are available concerning the amounts of hydroxyproline-peptides excreted during the conditions used when evaluating protein quality in rats. A

decreased utilization of the amino acids as nitrogen and energy source could occur if they were absorbed and excreted in the form of peptides.

This study was undertaken in order to establish the level of free and peptide bound hydroxyproline excreted in the urine of rats fed supplemented hydroxyproline-rich diets under conditions used during nitrogen balance experiments.

MATERIAL AND METHODS

Materials

Beef, *M. psoas major*, was freed of visible fat and connective tissue, minced in a Moulinex blender and lyophilized. Gelatin (swine skin, 175 Bloom), and insoluble collagen (bovine Achilles tendon, prepared according to Einbinder & Schubert (13)), were purchased from Sigma Chem. Comp., USA.

Pigskin was taken from a 6 month old pig which had been scalded according to slaughterhouse procedures. The pigskin was freed of visible fat and meaty parts, ground to pass a screen of 2 mm and lyophilized. Before being mixed into the diet, the pigskin was mixed with a portion of maize starch to avoid heating and ground in a hammer-mill passing a screen of 0.8 mm.

Rat assay

Nitrogen balance studies were performed on growing rats. Male Sprague-Dawley rats (initial weight 85 g) from Anticimex, Sweden, were kept individually in metabolic cages according to Eggum (14). Each diet was tested on five rats. A four day adaption period was followed by a five day balance period.

The diets tested contained protein in the form of meat, insoluble collagen, gelatin and pigskin. ANRC Reference Protein High Nitrogen Casein (Sheffield Chem., USA) was used as reference, supplemented with 0.22% methionine (dry matter). The meat, insoluble collagen and gelatin diets (0.78% nitrogen, dry matter) were mixed with fat extracted lyophilized whole hen's egg (0.72% nitrogen, dry matter). The pigskin diet was supplemented with 0.15% methionine and 0.10% each of tryptophan, isoleucine and histidine (dry matter). L-methionine (Biochemische Zwecke, Merck), L-tryptophan (chromatographically homogeneous) L-isoleucine and L-histidine x HCl (all from BDH Chem. Ltd, England) were used for supplementation.

All diets contained 1.5% nitrogen (dry matter). The contents of minerals and vitamins were modified as according to Forsum *et al.* (15). Maize starch was used as the carbohydrate source and to adjust dry matter content. Egg protein (0.72% of dry matter) was used for the determination of metabolic and endogenous nitrogen excretion.

The urine was collected in a water solution of Terramycin^R (Pfizer Inc., USA), 0.08 mg/ml and the faeces in 5% H₂SO₄. A drug, omeprazole, was administered to the rats given the diets containing meat/egg, gelatin/egg and insoluble collagen/egg, in order to inhibit the gastric acid secretion. The purpose was to study the importance of gastric acid for collagen and gelatin digestion as described separately by Laser Reuterswård & Fabiansson (1984, submitted for publication). The

diets containing casein and pigskin were given without omeprazole. Calculations of true digestibility (pigskin diet only) and biological value (all diets) were performed using the Thomas-Mitchell equations as described by Eggum (14).

Chemical analyses

Nitrogen content was determined using the Kjeldahl method.

Free hydroxyproline was analyzed directly from aliquots of urine. Total hydroxyproline was analyzed after the freeze-drying of 50 ml urine solution followed by hydrolysis in 10 ml of 6 M HCl at 110°C for 16 hours. Hydroxyproline was analyzed on a Technicon Auto-Analyzer according to Stegemann (16) as modified by Weber (17). The content of peptide bound hydroxyproline was calculated as the difference between total and free hydroxyproline (9). Calculations of collagen content in the diets were made according to Laser Reuterswärd *et al.* (18).

Statistical evaluation

Means and standard deviations were calculated and analyzed by Student's t-test (19).

RESULTS AND DISCUSSION

The urinary excretion of hydroxyproline (HPRO) in rats fed the diets of differing HPRO-content are shown in Table 1. The excretion of total and free HPRO in rats fed the meat diet was not statistically different from the corresponding values using the casein control. However, with the diets containing insoluble collagen, gelatin and pigskin, the excretion of total and free HPRO was significantly higher ($P < 0.05$).

The ratio between free and total HPRO was found to be between 0.32 (casein diet) and 0.79 (pigskin diet).

Corrections for basal excretion of total and free HPRO, by subtracting HPRO values of the HPRO-free casein diet from the HPRO-rich diets, were made. Data in Table 1 show that after these corrections, the total HPRO-values were equal to the free HPRO-values in each of the three HPRO-rich diets and a constant difference was obtained between total and free HPRO (uncorrected). This indicates that the excretion of peptide-bound HPRO did not increase after ingestion of insoluble collagen, gelatin and pigskin. Thus, with increasing HPRO-content in the diet, there was an increase in the urinary excretion of free HPRO only. The total excretion of HPRO from rats fed the most HPRO-rich diet, the pigskin, corresponded to 2.5% of the ingested HPRO.

For the HPRO-free casein-diet, the total amount of HPRO excreted, when calculated as an average of five days excretion, corresponded to 0.7 mg/24 hours, and the free amount of HPRO was 32% of the total amount. The first value is similar but the second one is somewhat higher than the levels previously reported in Sprague-Dawley rats fed soy protein or casein diets (20, 11).

The total and free HPRO excreted by the rats fed the casein control was considered as a measure of the basal degradation of tissue collagen. The relevance of this assumption was indicated by Prockop & Sjoerdsma (9), who showed that

Table 1. Urinary excretion of hydroxyproline (HPRO) in rats ($n=5$) fed diets with different HPRO-contents, collected from a five-day nitrogen balance period. HPRO was estimated before (free) and after (total) acid hydrolysis of the urine. Standard deviations within brackets.

	Dietary Nitrogen (dry matter)	Dietary HPRO	Total HPRO in urine		Free HPRO in urine	HPRO-Difference Total-free		Biological Value
			uncorrected	corrected ¹⁾	uncorrected	corrected ¹⁾	(uncorrected)	
	g/100g	mg/gN	mg/5d	mg/5d	mg/5d	mg/5d	mg	%
Casein supplemented ²⁾	1.50	Zero	3.39 (0.50)	0.00	1.03 (0.46)	0.00	2.36	91.8 (3.1)
Meat/Egg	0.78/0.72	11	3.25 (0.45)	-0.14	1.26 (0.45)	0.23	1.99	94.9 (1.0)
Insoluble Collagen/Egg	0.78/0.72	370	4.84 (1.30)	1.45	2.47 (0.90)	1.44	2.37	80.5 (2.9)
Gelatin/egg	0.78/0.72	371	7.75 (2.88)	4.36	5.12 (2.60)	4.09	2.63	72.5 (3.1)
Pigskin supplemented ³⁾	1.50	632	10.76 (4.32)	7.37	8.73 (4.00)	7.70	2.03	52.6 (1.8)

¹⁾ Corrected value = average value with the test diet minus average value with the casein diet

²⁾ Supplemented with 0.22 % methionine (dry matter)

³⁾ Supplemented with 0.45 % of essential amino acids (dry matter)

humans excreted a constant amount of peptide-bound HPRO. This excretion of hydroxyproline peptides is thus used as an index of basal collagen degradation (21). The rats fed the meat diet (containing very low amounts of HPRO) did not excrete higher levels of total or free HPRO than those fed the casein diet. This result supports the findings concerning basal degradation of collagen.

In the present study it was found that the elevation in HPRO excretion after ingestion of HPRO-rich diets was mainly as free imino acid. This does not agree with Hueckel & Rogers (11) who reported that in rats both peptide-bound and free urinary hydroxyproline increased when the diet contained gelatin. Furthermore, data on normal humans also showed that after ingestion of 25 g gelatin the increased urinary excretion was mainly in the form of peptides, amounting to about 7% of the HPRO in the diet (9, 12, 22).

Weiss (7) reported that the unusual amino acid sequences of denatured collagen would make it a poor substrate for the exo- and endopeptidases. It is, however, generally stated that one physiological role of the intestinal mucosal cytosol peptide hydrolases is to catalyze the intracellular degradation of protein and peptides including, for example, collagen fragments (23). Proline containing dipeptides or tripeptides are thus not readily hydrolyzed by the brush boarder enzymes, but are preferentially degraded in the cytoplasmic fraction. The two dipeptides of proline and glycine are hydrolyzed but at a relatively low rate in the rat intestine. These dipeptides have been detected in superior mesentric venous blood after intraluminal injection into rats (24). Prolidase, which has been shown to occur in intestinal mucosa and is the only known enzyme cleaving proline-hydroxyproline, hydrolyzes the peptide very slowly (25). The enzyme glutamic acid-leucine dipeptidase, isolated from, for example, the Sprague-Dawley rat (23) appears to hydrolyze some of the iminodipeptides (proline-x) effectively, but the number of proline-containing dipeptides studied are small (8). Thus it appears

that there are dipeptidases which can hydrolyze peptides containing proline and hydroxyproline in the rat intestine. A difference in the HPRO-rich protein dosage might explain the discrepancy between the excretion of free imino acid found in our study and peptide bound HPRO found by others (11, 9, 12, 22). We used a low (9%) total protein content in the diets. A higher protein and HPRO-content might have saturated HPRO-peptide hydrolysis in the intestine with possible absorption and excretion.

The biological value of the five diets is shown in Table 1. A significantly higher biological value and lower urinary HPRO was obtained with the insoluble collagen/egg diet than with the gelatin/egg diet. This can be explained by the lower digestibility of collagen (71%) than of gelatin (98%) after inhibition of gastric acid secretion (Laser Reuterswärd & Fabiansson, 1984, submitted for publication). This results in a higher proportion of absorbed amino acids from egg in the mixture of insoluble collagen/egg. Meat proteins were 98% digestible (Laser Reuterswärd & Fabiansson, 1984, submitted for publication) and pigskin proteins were found to be 98% digestible.

In conclusion, hydroxyproline-rich diets, supplemented with egg protein or amino acids, gave elevated excretion of hydroxyproline. After correction for basal excretion of hydroxyproline, the excretion determined was in the form of free imino acid only. At most, 2.5% of the ingested hydroxyproline was excreted by the rats. The urinary losses therefore do not substantially diminish the utilization of amino acids as nitrogen or energy source.

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Digestibility of collagenous fermented sausage in man

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SUMMARY

Protein digestibility of a fermented collagenous sausage was studied in three ileostomists with small bowel resections. The patients were given four ordinary meals each day with a total protein content of about 64 g, half of which was derived from collagen. Pigskins from 6 month old scalded pigs were used as the collagen source. A fermented sausage based on meat was used as reference, and the ileostomists served as their own controls. Urine was collected by two patients. The true nitrogen digestibilities were found to be 71-79% for the collagenous diets and 69-85% for the reference diets. Hydroxyproline digestibilities (apparent and true) for the collagenous diet periods were similar, 70-82%. This indicated that collagen was digested to the same extent as other proteins. Amino acid patterns of ileal excreta distinctly differed for each of the two periods, further confirming that all protein was not absorbed. Elevated excretion of hydroxyproline in the urine after ingestion of the collagenous sausage was found, mainly as peptide bound hydroxyproline, but accounted for about only 2% of the ingested hydroxyproline. It was concluded that collagenous fermented sausage is as digestible as a reference sausage based on meat even without prior heat treatment.

INTRODUCTION

Protein of collagenous origin normally constitutes a small part of food proteins. However, in some products, as for example Swedish sausages, up to 35% of the protein can be of collagenous origin (Fuchs & Kuivinen, 1980). The content of intramuscular collagen varies from about 2 to 20% of the protein (Lawrie, 1979). In sausages about 50% of the collagen is derived from pigskin and from back fat with or without pigskin. Different opinions exist concerning the digestibility of collagen. Brüggeman *et al.* (1964) stated that mammals can digest native collagen while other authors consider the fibers indigestible (Loewit 1970, Cheftel 1977; 1979; von Hofsten, 1978; Kies 1981). However, solubilization and denaturation to gelatin, as e.g. after cooking, makes collagen digestible (Paul 1972; Rogowski, 1980; Ashgar & Henrickson, 1982).

Two aspects could be especially critical for digestibility of the collagen molecule. Firstly, collagen from tissues as e.g. pigskin and tendon is mainly insoluble (Johns, 1977; Carmichael & Lawrie, 1967). Insoluble collagen must be solubilized before

denaturation and pepsin can to a large extent solubilize collagen (Etherington, 1977). Accordingly, in an earlier study the protein in native pigskin (75% collagen) and tendon (95% collagen) was found to be highly solubilized after incubation with pepsin and pancreatin (Laser Reuterswärd, 1984). Secondly, the unusual amino acid pattern of denatured collagen (gelatin) could make it a poor substrate for exo- and endopeptidases (Weiss, 1976). Chen *et al.* (1962) indicated that gelatin has a slow or poor digestibility in rats. However, Mauron (1973) found gelatin 90% digestible in rats and in a previous study of ours more than 92% of the protein in pigskin and tendon, from young as well as old animals, was digestible in rats even without prior heat treatment (Laser Reuterswärd *et al.*, 1984).

No study is available where digestibility of collagen or gelatin has been directly measured in man. However, Prockop *et al.* (1962) showed that up to 7% of hydroxyproline in gelatin was excreted as peptides in human urine due to the absorption of intact peptides. Kofranyi & Jekat (1969) studied the biological value of a mixture of gelatin and meat without specifically discussing digestibility.

A higher pH during pepsin digestion of pigskin and tendon resulted in less solubility *in vitro* (Laser Reuterswärd, 1984). Inhibition of gastric acid secretion in rats by using a drug significantly decreased the true digestibility of insoluble tendon collagen (Laser Reuterswärd & Fabiansson, 1984). The pH in the stomach during digestion in humans varies individually and with the composition of the diet (Rogers & Harper, 1966; Davenport, 1977).

This study was undertaken in order to study the protein digestibility of a highly collagenous sausage given to subjects with ileostomy in ordinary meals. Pigskin was used as a natural collagen source and fermented sausage as a model for processing without heat treatment. As the study was performed in ileostomists with different lengths of small bowel resection - and presumably with varying degrees of malabsorption - each individual served as their own control.

SUBJECTS, MATERIALS AND METHODS

Subjects

Three ileostomists, two women (33 years, 41 years) and one man (27 years), volunteered to take part in the study. The patients were operated due to Crohn's disease and distal parts of the small bowel had been resected (50; 70 and 120 cm, respectively). All patients had well established ileostomies with rather constant volume losses.

The study was approved by the Ethical Committee of Sahlgren's Hospital.

Experimental model

Each morning during the study, the patients attended the out-patient clinic, ate the morning meal, received food prepared in the metabolic kitchen for the rest of the day, a container for ileostomy bags and urine bottles. After the first day bags and bottles used the day before were returned for analyses.

The study of each patient extended over a period of 4 days, 2 days of collagenous diet and 2 days of reference diet, starting at breakfast time. Patient 1 ate collagenous diet for 2 days and after 14 days ate the reference diet for 2 days. Patient 2 and 3 ate the diets over four consecutive days.

It has been shown in a previous study that ileal excreta collected between 8 a.m. one day and 8 a.m. the next day corresponded to all the food ingested during that 24 hour period. (Sandberg *et al.* 1981).

Sausage preparation

Pigskins were collected from 6 month old pigs that had been scalded in water at 60°C for 10 minutes according to slaughterhouse procedures. The pigskins were freed of visible fat and meaty parts, cut in a bowl chopper for some minutes and thereafter ground together with a portion of ice passing a screen of 2 mm.

Beef, *M. longissimus dorsi*, and ham were freed of visible fat and connective tissue. Pork back fat was freed of meaty parts. The raw products were collected from a slaughterhouse.

Batches, of about 5 kg were made of two collagenous sausages and two reference sausages. The sausages were prepared from pigskin (collagenous sausage only), meat, pork back fat, ice, cooked potatoes, whole blood (bovine), spices (white pepper, black pepper and onion powder), salt (0.6% nitrite), locust bean gum (stabilizer, Frimulsion 10, Hercules, Sweden) and Culcid (a starter culture of *Pediococcus*, 10⁸, Culinar, Sweden) in proportions shown in Table 1. The ingredients were mixed in a bowl chopper, the final temperature of the batter was being at a maximum of 18°C. The batter was put into casings (Visco, Kontrollhudar, Sweden) with a diameter of 55 mm. The sausages were smoked for four hours at a wet temperature of 24°C, 90% relative humidity and dried for 13 days at the same temperature and humidity. They were then washed with 2% sorbic acid, packed in vacuum packages and stored at +1°C, for up to 3 months. The casings were removed before consumption. After mincing samples in water the pH was estimated to be 4.6 for all sausages. The composition of the fermented sausages is shown in Table 2.

Table 1. Composition of sausage batter recipe for collagenous and reference sausages. Protein and collagen contents are calculated in % of total protein and total collagen.

Ingredients	Collagenous batter				Reference batter			
	Contents	Protein			Contents	Protein		
	g	g	% of total protein	% of total collagen	g	g	% of total protein	% of total collagen
Beef	875	350	35.1	4.0	3875	950	96.0	99.0
Ham	875				875			
Pork back fat	500	13	1.3	0.1	900	23	2.3	1.0
Pigskin	2050	615	61.9	95.9	0			
Water (ice)	500				0			
Potatoes (cooked)	500	8	0.8		500	8	0.8	
Whole Blood	50	9	0.9		50	9	0.9	
Salt (0.6 % nitrite)	125				125			
Spices	39				39			
Locust Bean Gum	20				20			
Culcid starter culture	10				10			
			100 %	100 %			100 %	100 %

Table 2. Composition of fermented sausages

	Collagenous %	Reference %
Water	42.7	43.3
Protein (collagen)	27.6 (15.3)	28.0 (1.4)
Fat	22.5	21.4
Ash	5.1	4.8

Diets

Protein 'free' bread and macaroni were used in order to minimize protein intake from other sources than the sausages.

Breakfast consisted of 25 g sausage and bread (made from a protein 'free' mix, JUVEL AB, Sweden), lunch of 75 g sausage and macaroni salad (Aglutella, Gentili-Divisione, Dietetici, Italy), dinner of 75 g sausage and mashed potatoes, and the evening meal identical to breakfast. All the sausages were consumed without any heat treatment. Bread and muffins (made from the protein 'free' mix as above) and bread made of Aprotein Biscottato (Astra Meditec, Sweden) were consumed as a free addition by the patients. The diet contained about 64 g protein daily and its composition is shown in Table 3.

The energy levels of diets for the three patients were calculated to be 2000, 3000 and 2500 kcal, respectively, distributed as: protein 12, 8 and 10%; fat 45, 41 and 41% and carbohydrates 42, 46 and 46%, respectively.

Duplicate portions of the diets were collected on the second and forth days and the samples were homogenized after the addition of 10 ml emulsifier (disodiumochtylsulphosuccinate). The mixtures were freeze-dried and the powders were ground by hand using a mortar and pestel.

Collection of ileostomy contents

The ileostomy bags were changed every 2nd hour daily from morning until bedtime and once during the night. The bags were immediately frozen on dry ice to avoid bacteriological degradation, weighed and stored at -20°C. Each bag with its contents was then separately freeze-dried and the powder ground by hand using a mortar and pestel and the weight estimated. Proportions of powders for every 24 hours were pooled and ground as above.

Urine collection

Urine was collected from patients no 2 and 3. Day 1, morning urine was discarded and the urine until bedtime was collected as day urine. Night urine was collected the next morning. Thus, over four days a total of 8 samples per patient was collected. The urine was kept for a maximum of 24 hours at +10°C and thereafter frozen. After thawing, the volume was estimated, aliquots were refrozen and stored at -20°C until analyzed.

Table 3. Daily Diet Composition

	Wet weight g	Protein ¹⁾ g
Sausage	200	55
Protein 'free' bread	105	0.6
Extra protein 'free' bread and muffins	0—240	<1.5
Maccaroni	60—140	<0.5
Mashed potatoes with cream	150	1.4
Margarine	14—30	trace
Marmalade	25	—
Mayonaisse	35	0.4
Cucumber, pickled	20	0.2
Common beet, pickled	20	0.2
Leek	3	trace
Lettuce, tomatoes, cucumber	30—150	1.4
Lemon water	4 + 16	—
Salt	2—3	—
Beer or soft drink	60—120	trace
Tea or coffee	0—30	—
Water	970—2500	—

¹⁾ The Sausage value is analytical, other values being from food composition tables (the Swedish National Food Administration, 1978) or from figures given by the manufacturer.

Analytical methods

Wet weight and dry weight of freeze-dried ileostomy contents were calculated from the weights before and after freeze-drying. **Dry weight** was estimated after drying 1 g at 104°C overnight. **Ash** weight was determined after dry-ashing powders overnight at 550°C according to the Nordic Committee on Food Analysis, no 23, 1974. **Dietary fiber** was determined gravimetrically using the method of Asp *et al.* (1983). Values were corrected for indigestible protein and ash associated with the fiber. Free **glucose** was determined with a glucose oxidase reagent (Glox-Novum, Kabi, Sweden). **Starch** was determined as glucose liberated by incubating boiled suspensions of ileostomy evacuate with amyloglucosidase (Boehringer Mannheim, GFR) at pH 4.5, 60°C for 30 min, and expressed on anhydro basis (glucose×0.9). Total **fat** and **fatty acids** of the ileostomy contents were determined according to Van de Kamer *et al.* (1949). **Nitrogen** was determined on sausages, diets, ileal excreta and urine using the Kjeldahl method. **Hydroxyproline** (HPRO) in sausages was determined according to Woessner (1961) (method 1) and in diets, ileal excreta and urine according to Kivirikko *et al.* (1967). Hydrolysis was performed with 6 M HCl at 110°C for 16 hours. Urine was analyzed before (free HPRO) and after (total HPRO) hydrolysis and the content of peptide bound hydroxyproline was calculated to be the difference between these two values as according to Prockop & Sjoerdsma (1961). Calculations of collagen content of the protein were made as described by Laser Reuterswärd *et al.* (1982). **Amino acid** analyses were performed in duplicates after acid hydrolysis in 6 M HCl at 110°C for 24 hours. Cystine and methionine were determined after performic acid oxidation. Separation was performed on a Durrum D-500 Analyzer at the Central Amino Acid Analysis Laboratory, Biomedical Centre, Uppsala, Sweden.

Statistical evaluation

Means and standard errors were calculated and analyzed by Students' t-test for small samples and unknown variances assumed equal (Bailey, 1981).

RESULTS

Diets

Table 1 shows that 96% of the collagen in the collagenous sausage derived from the pigskin. The small amount of collagen in the reference sausage was derived from intramuscular collagen in beef and ham. As shown in Table 2 the protein, fat and water content of the two sausages were similar. The sausages accounted for 79-86% of the total dietary protein (Table 3, 5). Potatoes and vegetables were the main sources of the remaining protein.

Ileostomy contents

Table 4 shows the compositions of ileostomy contents. A tendency to slightly higher values for all components in the collagenous samples compared to the reference can be seen. A statistically significant ($P < 0.05$) difference was found for ash content (paired t-test) but all other differences were insignificant ($P > 0.05$) when using t-test and paired t-test. The components analyzed accounted for a mean of 102% of the dry matter for the collagenous period as well as the reference period when assuming a conversion factor of nitrogen to protein of 5.9 (Table 4).

Nitrogen and hydroxyproline excretion

Table 5 shows nitrogen and hydroxyproline balances. Values of 78-85% of the total dietary nitrogen derived from sausages. Almost half of the protein being derived from collagen in the collagenous diet period, and only 5% in the reference diet period. The total intake of nitrogen for different patients and periods was kept at the same level. Statistical evaluation of nitrogen excretion showed no significant difference between day 1 - day 2 for period 1 and period 2, respectively, or for period 1 compared to period 2 (paired t-test). Thus, no significant difference was found between nitrogen excretion in collagenous periods when compared with reference periods ($n=6$, t-test).

The average excretion (24 hours) of hydroxyproline was for the collagenous periods $1.15 \text{ g} \pm 0.10$ (mean $\pm$ SEM) compared to $0.16 \text{ g} \pm 0.02$ for the reference periods ($P < 0.001$).

Calculations of apparent digestibility (AD) and true digestibility (TD) from the nitrogen and hydroxyproline excretion are shown in Table 5. TD-values of nitrogen were 70.5-78.5% for the collagenous diet periods and 68.8-84.9% for the reference diet periods when assuming a basal nitrogen excretion of 0.8 g/day (Sandberg, 1982). Hydroxyproline digestibilities (AD and TD) for collagenous diet periods were similar (70.0-81.5%) indicating similar digestibility of collagen and other protein. The hydroxyproline digestibility (AD) for the reference diet period indicated that intramuscular collagen had a digestibility of at least 51-72%.

Table 4. Compositions of ileal excreta (g/24h) for three patients eating diets over a two day period (n=6). Mean values with their SEM.

	Collagenous			Reference		
	Mean	SEM	Range	Mean	SEM	Range
Wet weight	1426	55	(1316—1683)	1296	86	(1008—1605)
Dry weight	79	5	(68—106)	72	5	(56—83)
Nitrogen	3.56	0.26	(2.93—4.53)	3.17	0.37	(2.3—4.50)
Protein (N × 5.9)	21.0			18.7		
Ash	12.6	0.47	(11.6—14.7)	11.5	0.65	(9.4—13.8)
Fiber	21.4	2.76	(17.5—34.7)	19.6	1.58	(16.0—24.9)
Starch	4.3	0.36	(2.8—5.3)	3.9	0.18	(3.4—4.6)
Glucose	1.9	0.38	(0.9—3.2)	1.7	0.27	(0.8—2.8)
Fatty acids	18.0	1.22	(15.7—23.8)	16.6	1.19	(12.7—19.0)
Fat	2.1	0.22	(1.6—3.1)	2.0	0.21	(1.2—2.4)

Table 5. Nitrogen and hydroxyproline balance. Composition of diet and ileal excreta/24h.

Patient	Diet-Day	NITROGEN						HYDROXYPROLINE			
		Daily diet		Ileal excreta	Digestibility			Daily diet		Digestibility	
		g	% from sausage		g	AD ¹⁾ %	TD ²⁾ %	g	Ileal excreta g	AD ³⁾ %	TD max ⁴⁾ %
1	Col-1	10.98	85.5	47.1	3.15	71.2	78.5	4.05	1.14	73.8	76.6
	Col-2				3.16				0.978		
	Ref-3	10.35	85.1	5.2	2.31	77.2	84.9	0.364	0.125	69.4	
	Ref-4				2.36				0.098		
2	Col-1	11.91	78.8	43.4	2.93	70.5	77.3	5.20	0.840	78.5	81.5
	Col-2				4.09				1.39		
	Ref-3	11.94	79.2	4.3	2.79	75.4	82.1	0.556	0.151	71.7	
	Ref-4				3.08				0.164		
3	Col-1	10.96	81.8	41.9	3.51	63.2	70.5	4.30	1.12	70.0	74.9
	Col-2				4.54				1.45		
	Ref-3	11.04	85.7	4.6	4.50	61.5	68.8	0.430	0.216	51.3	
	Ref-4				4.00				0.203		

¹⁾ AD = Apparent digestibility = $((N_{intake} - N_{excretion}) / N_{intake}) \times 100$

²⁾ TD = True digestibility = $((N_{intake} - (N_{excretion} - N_{basal\ excretion})) / N_{intake}) \times 100$; calculated with basal excretion of 0.8 (Sandberg, 1982)

³⁾ AD as above but with hydroxyproline

⁴⁾ TD as above but with hydroxyproline; calculated assuming basal excretion of hydroxyproline equivalent to excretion on reference sausage and equivalent to maximal TD = TD max

Amino acid composition

Table 6 shows the amino acid composition of ileal excreta. Similar compositions (with small SEM) were obtained for the three patients during both the collagenous period and the reference period. Higher amounts of hydroxyproline, proline and glycine were found after the collagenous periods. The same levels were found for serine, alanine, tyrosine and histidine but the 10 other amino acids analyzed occurred in larger amounts in the samples from the reference periods. During the collagenous diet periods the proportion of hydroxyproline/proline was 0.62, hydroxyproline/glycine 0.41 and proline/glycine 0.67. These values are similar to the calculated proportions of amino acids in the collagenous sausage.

Urine excretion

Table 7 shows nitrogen and hydroxyproline excretion for two patients. They excreted 33 and 28% more nitrogen, respectively, after eating the collagenous sausage than after eating the reference sausage. Elevated amounts of hydroxyproline were also found (Table 7), and 74% and 91% of this increase was peptide bound. The ratio of hydroxyproline/nitrogen in urine was quite constant over the different periods (two days, two nights, $n=4$), and higher for the collagenous period.

DISCUSSION

The present study shows that the digestibility of fermented sausage, with about 45% of the protein from collagen, was as high as that of the reference sausage. The true protein digestibilities of both types of sausage were calculated to be 75-80%. Fasting ileostomists without small bowel resection have nitrogen losses of about 0.8 g/day (Sandberg, 1982) and this figure was used for the TD-calculations (Table 5). Such patients lose only about 2 g of nitrogen daily on a protein-rich meat based diet (Sandberg *et al.* 1981) implying a TD of about 90%. Patients with ileal disease or after ileal resection with remaining colon, lose a mean of about 4 g of nitrogen per day on a mean intake of 95 g of protein (Andersson *et al.* 1974). The rather low digestibility of meat protein in the reference period found in the present study can thus probably be ascribed to some malabsorption of the surgically resected patients. Moreover, patients 1 and 2 previously showed AD values of about 75% for a normal protein diet and about 66% for a soya diet, values that were lower than those observed in other ileostomists without small bowel resections (Andersson, unpublished).

It was previously shown that pigskin from a scalded 6 month old pig and native pigskin from a 4 month old pig were highly solubilized by pepsin and pancreatin *in vitro* (83 and 94%, respectively) (Laser Reuterswärd, 1984); and both had a true digestibility of 97% in rats (Laser Reuterswärd *et al.* 1984). The results of the present study are in accordance with these observations and confirm that unsolubilized and undenatured collagen derived from pigskin is equally digestible in man as meat protein even without prior heat treatment. The probable mechanisms for digestion of collagen in the gastrointestinal tract are discussed in more detail elsewhere (Laser Reuterswärd, 1984; Laser Reuterswärd & Fabiansson, 1984).

Table 6. Amino acid composition (g/18 g N corresponding to 100 g protein + SEM) of ileal excreta; collagenous period day 2 and reference period day 4.

	Collagenous period % $\pm$ SEM g/18 g N n = 3	Reference period % $\pm$ SEM g/18 g N n = 3
Aspartic acid	9.63 $\pm$ 0.34	10.60 $\pm$ 0.16
Threonine	4.76 $\pm$ 0.16	5.64 $\pm$ 0.18
Serine	5.32 $\pm$ 0.20	5.61 $\pm$ 0.27
Glutamic acid	11.93 $\pm$ 0.21	13.74 $\pm$ 1.10
Proline	9.42 $\pm$ 0.45	6.08 $\pm$ 0.33
Glycine	14.04 $\pm$ 0.21	8.91 $\pm$ 0.71
Alanine	5.31 $\pm$ 0.09	5.08 $\pm$ 0.31
Cystine	1.95 $\pm$ 0.25	2.64 $\pm$ 0.16
Valine	4.31 $\pm$ 0.13	5.29 $\pm$ 0.13
Methionine	0.88 $\pm$ 0.07	1.26 $\pm$ 0.18
Isoleucine	2.97 $\pm$ 0.13	4.07 $\pm$ 0.41
Leucine	5.06 $\pm$ 0.16	6.60 $\pm$ 0.57
Tyrosine	3.03 $\pm$ 0.06	3.90 $\pm$ 0.10
Phenylalanine	3.28 $\pm$ 0.10	4.09 $\pm$ 0.20
Histidine	2.13 $\pm$ 0.04	2.67 $\pm$ 0.13
Lysine	4.40 $\pm$ 0.09	6.05 $\pm$ 0.74
Arginine	4.42 $\pm$ 0.23	4.83 $\pm$ 0.56
Glucosamine	5.42 $\pm$ 0.64	5.22 $\pm$ 1.39
Galactosamine	2.52 $\pm$ 0.29	1.38 $\pm$ 0.70
Ammonia	2.53 $\pm$ 0.08	2.80 $\pm$ 0.14
Hydroxyproline	5.82 $\pm$ 0.16	0.87 $\pm$ 0.06

Table 7. Urinary excretion of nitrogen and hydroxyproline (HPRO).

Patient	Diet	Nitrogen g/48 h	Total HPRO mg/48 h	Free HPRO mg/48 h	Total HPRO/Nitrogen % (SEM) n = 4
2	Collagenous	17.1	226.0	74.0	1.32 (0.02)
	Reference	12.9	66.5	31.8	0.51 (0.03)
	Difference		159.5	42.2	
3	Collagenous	15.6	135.6	23.4	0.87 (0.06)
	Reference	12.2	44.4	14.8	0.37 (0.13)
	Difference		91.2	8.6	

The similar amino acid pattern obtained in the ileal excreta after the collagenous period as in the collagenous sausage indicated a similar digestibility of different protein components, i.e. collagen was not enriched in the ileal excreta. The amino acid pattern in ileal excreta of the two periods distinctly differed.

Pigskin was used as a natural source for collagen but had to be scalded for practical and microbiological reasons before consumption. Fermenting was used to give an acceptable palatability without further heat treatment. Fermented sausages with low collagen contents have been shown to be 93% digestible in rats (Eskeland & Nordal, 1980). However, the way of processing could have influenced the collagen fibers in different ways. Scalding may denature some of the soluble collagen in immature pigs (Johns & Courts, 1977). However, about 90% of collagen in skin is insoluble (Johns, 1977) and insoluble collagen denatures at temperatures of about 65°C (Bailey, 1968), the exact shrinkage temperature for pigskin is not available in literature. Scalding could thus only have denatured a small part of the pigskin collagen. Fermentation induces a high microbiological activity which could result in proteolytic activity. The main flora developed in the sausages was the starter culture of *Pediococcus* and this species does not liquify gelatin i.e. it is non-proteolytic (Kitahara, 1974) and therefore has no collagenolytic activity. Another possibility of enzymatic action on collagen fibers could be the solubilization by cathepsins. These enzymes are present in muscle cells (Dutson, 1982) and could be derived from meat mixed into the collagenous sausage. Cathepsins can solubilize different collagen fibers at 25°C in solution and at low pH (3.4) (Etherington, 1977). However, the structure of the sausage was crude and water content relatively low. Therefore diffusion of enzymes and degradation of pigskin collagen does not seem very probable. Moreover, the pH of 4.6 in the sausages would probably be too high since insoluble collagen is totally resistant to degradation by e.g. cathepsin B above pH 4.5, probably due to the lack of swelling, and cathepsin D has no solubilizing action on fibrous collagen (Bailey & Etherington, 1980). Fermentation of the collagenous sausage at 25°C to pH 4.6 should therefore not have solubilized collagen fibers.

Urinary excretion of hydroxyproline

It has previously been shown that peptide bound hydroxyproline is the form of hydroxyproline that predominates in human urine as a consequence of basal collagen degradation (Prockop & Sjoerdsma, 1961; Meilman *et al.* 1963). In the present study the hydroxyproline excreted in the reference period was peptide bound to 52 and 67%, respectively. These values are somewhat lower than previously reported. After the consumption of the collagenous sausage the elevated excretion of hydroxyproline was, however, mainly in peptide bound form. This agrees with earlier reports where normal humans ingested 25 g gelatin (Prockop & Sjoerdsma, 1961; Prockop *et al.* 1962; Bronstein *et al.* 1966), resulting in an excretion of 7-8% of the total hydroxyproline ingested (Prockop *et al.* 1962). These values are somewhat higher than the values of 2.2 and 1.6%, respectively, found in the present study.

CONCLUSION

In conclusion, the present study on ileostomists with small bowel resection showed that digestibilities of 70-80% were obtained for the collagenous sausage and likewise for the reference sausage based on meat. Even in malabsorptive states collagen was thus absorbed to the same extent as meat protein. The fermented sausage was used as a model sausage without heat treatment showing that prior conversion to gelatin is not necessary for the digestion of insoluble collagen in the gastrointestinal tract of man.

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